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Taxis transducer proteins and complex pattern formation by *Azotobacter vinelandii* UW

by

Heather Lynn Whelan

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requirements for the degree of Master of Science

in

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DEDICATION

This thesis is dedicated to my parents Lloyd and Patricia. I am so grateful for your support during my university odyssey and I will joyfully spend the remainder of my life repaying you for your generous gift of an education.

ABSTRACT

Taxis transducer proteins are the sensory component of bacterial taxis systems. *Azotobacter vinelandii* are motile, diazotrophic cells that are found in soil and freshwater habitats. Bioinformatic analysis of the *A. vinelandii* genome identified nine transducer genes: *aerP, aerH, aerB, tlpA, tlpB, tlpC, tlpD, tlpE* and *tlpF*. The *aerP* and *aerB* genes encode a N-terminal PAS domain that may be involved in sensing intracellular energy levels. All the transducers were disrupted by insertional mutagenesis and the motility of the transducer mutant were examined on soft agar media. The a*erB*, but not *aerPH*, mutants produced significantly smaller halos than the wild type cells on all motility substrates tested. The *tlpB* mutants were also impared on all substrates tested. The soft agar experiments also lead to the discovery of complex pattern formation by motile *A. vinelandii*. The genes *aerB*, *tlpA*, *tlpB* and *tlpC* but not *aerP*, *aerH*, *tlpD*, *tlpE* or *tlpF* are essential for pattern formation.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BBGN	Burkes buffer containing glucose and ammonium acetate
bp	base pair(s)
BSA	bovine serum albumin
CCW	counter-clockwise
CW	clockwise
Cyt	cytochrome
DH	dehydrogenase
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DTT	1,4 dithiothreitol
Δp	proton motive force
EDTA	ethylenediamine tetraacetic acid
e^-	electron
$FAD(H_2)$	flavin adenine dinucleotide (the H is present in the reduced form)
H^+	hydrogen ion, or proton
HCD	highly conserved domain
IPTG	isopropyl β-D-thiogalactopyranoside
kDa	kilodalton
Km	michaelis constant
Km	kanamycin

l	liter
M	molar
Mb	mega base-pair
MCP	methylated chemotaxis protein
MH	methylated helices
ml	milliliter
mM	millimolar
MOPS	4-morpholinopropanesulfonic acid
ms	millisecond
mV	millivolts
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
NAD(P)H	β-nicotinamideadenine dinucleotide (phosphate) (the H is present in the reduced form)
NDH	NAD(P)H dehydrogenase
ng	nanogram
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PHB	poly-β-hydroxybutyrate
Pi	inorganic phosphate (PO_4^-)

pmol	picomole
PTS	phosphotransferase system
s	second
SDS	sodium dodecylsulphate
Sm	streptomycin
Spe	spectinomycin
Tc	tetracycline
TM	transmembrane
Tris	Tris(hydroxymethyl)-amino-methane
U	unit (of enzyme activity)
UV	ultraviolet
V	volt
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
χ~P	phosphorylated protein, where χ is the protein

CHAPTER 1

INTRODUCTION

1.1 Introduction

In response to environmental stress, bacteria are faced with essentially two choices: alter gene expression to deal with the environmental stress, or move away. It is this latter response that will be the focus of this thesis. Bacterial motility is a complex subject with a long history in the science of microbiology. The signal transduction pathway that governs motility has been elucidated in enteric organisms and to a certain extent in a handful of other bacteria. Despite these advances there are still many unanswered questions concerning the phenomenon of bacterial motility. In the past ten or so years there has been renewed interest in bacterial responses to oxygen (aerotaxis) and other energy-yielding compounds (energy taxis). In a complex natural habitat, such as soil, a diverse and heterogeneous environment constantly challenges bacteria. The notion is that energy taxis and aerotaxis will direct motile soil organisms to an optimal niche in their habitat. The organism of interest in this thesis is the soil bacterium *Azotobacter vinelandii*. This introduction will cover salient physiological characteristics of this organism followed by a review of bacterial motility and a review of the proteins involved in sensing tactic stimuli. Aerotaxis and energy taxis systems in other bacteria will also be discussed. Lastly, the phenomenon of complex pattern formation by motile bacteria will be described.

1.2 *Azotobacter vinelandii*

1.2.1 General characteristics of the organism

A. vinelandii is a free-living, motile, obligatory aerobic, Gram-negative, nitrogen-fixing bacterium (Kennedy *et al.*, 2001). Its cell morphology is generally rod- or ovoid-shaped and the cells are often arranged in singles or in pairs. Cell size is relatively large compared with other bacteria and *A. vinelandii* can have diameters of 1.5-2.0 μm or greater. *A. vinelandii* cells are motile via peritrichous flagella. There are many remarkable features of *A. vinelandii* that have made it an engaging subject of microbiological research for nearly a century. Some of these features include the ability to form desiccation-resistant cysts in response to stress. *A. vinelandii* also produces complex polymers such as bacterial alginate and poly-β-hydroxybutyrate (PHB), which serve a variety of functions for the cell and have potential industrial applications. Another notable feature of *A. vinelandii* is its high respiration rate, which is among the highest observed for any living organism (Robson & Postgate, 1980). The ability of *Azotobacter* species to fix nitrogen at high ambient oxygen concentrations is a rare feature among nitrogen-fixing prokaryotes and *Azotobacter* species have adopted several mechanisms to be able to fix nitrogen at such high oxygen tensions. In addition to these physiological characteristics *A. vinelandii* is also an interesting study from a molecular biology perspective. Certain strains are naturally competent, meaning they are able to take up extracellular DNA and the G+C% content of the genome is around 65%.

First discovered in 1903 in Vineland New Jersey by J. Lipman, *A. vinelandii* takes its name from the French noun *azote*, meaning nitrogen and the Greek *bacter* meaning rod or staff. *A. vinelandii* is a member of the family Azotobacteraceae, which includes the two genera *Azotobacter* and *Azomonas* (Kennedy *et al.*, 2001). These two genera are

differentiated on their ability to form cysts, where the latter genus is unable to form cysts. There is much diversity among *Azotobacter* species in traits such as motility, flagellar arrangement, pigmentation, carbon source utilisation and susceptibility to antimicrobial agents (Kennedy *et al.*, 2001). In terms of genetic relatedness to other organisms *A. vinelandii* shares many common characteristics with the opportunistic pathogen *Pseudomonas aeruginosa* (Reiders *et al.* 2004). *Azotobacter* species are widespread in nature and are found both in the soil and freshwater. For the most part *A. vinelandii* have been isolated from non-acidic soils although it has also been isolated from freshwater habitats (Thompson & Skerman, 1979). In order to understand the role that motility and tactic responses may play in the ecology of *A. vinelandii* a review of the relevant physiological characteristics of the organism is required.

1.2.2 Diazotrophy

Azotobacter species are able to fix atmospheric nitrogen (N_2) into ammonia (NH_3). A general overall reaction of biological nitrogen fixation is given below:

$$8e^- + N_2 + 8\,H^+ + 18\text{-}24ATP \rightarrow 2NH_3 + H_2 + 18\text{-}24ADP + 18\text{-}24Pi$$

The ability to fix nitrogen is restricted to certain species of eubacteria and archaea and to date no nitrogen fixing eukaryotic organisms have been identified. The ecological importance of biological nitrogen fixation cannot be underestimated; it is the only significant source of usable nitrogen for most of the organisms on this planet. Fixed nitrogen (*i.e.* ammonia, urea *etc.*) is ultimately lost to the atmosphere as N_2 because of the actions of denitrifying organisms. Therefore nitrogen fixation is the only major route to

recover the lost nitrogen. The enzyme responsible for biological nitrogen fixation is called nitrogenase; *A. vinelandii* possesses three distinct nitrogenases (Bishop, 1993). The differences among the three nitrogenase systems are the metal cofactors associated with the reactive centres of the protein complex. Nitrogenase I contains an iron-molybdate cofactor, nitrogenase II contains an iron-vanadium cofactor, and lastly nitrogenase III uses only iron as a metal cofactor. The expression of each of these nitrogenase systems is affected by the presence of different metal co-factors.

Expression of nitrogenase genes is controlled by the atypical two-component regulatory system NifL-NifA. This system is able to integrate metabolic signals of redox status, energy and fixed-nitrogen presence to control the expression of nitrogenase genes. NifA is a transcriptional factor that acts in conjunction with the alternative sigma factor RpoN (σ^N or σ^{54}) to activate transcription of nitrogenase gene promoters. The anti-activator NifL senses the various metabolic cues and will form a protein-protein complex with NifA, blocking NifA function, under conditions where diazotrophy is not necessary (Dixon, 1998). The factors that are sensed by NifL are shown in Figure 1.1. NifL is a flavoprotein capable of redox sensing, it has a redox potential of –196 mV at physiological pH (Dixon, 1998). If NifL becomes oxidized, due to high oxygen concentrations or lack of reducing equivalents, then it will form a complex with NifA preventing nitrogenase gene transcription. The cellular energy level can also be measured by the ratio of ADP/ATP. ADP bound NifL will inhibit NifA activity (Figure 1.1). It is believed that the regulation of NifL by ADP serves to prevent nitrogenase production when the cellular phosphorylation levels will not support the high energetic demands of nitrogenase (Dixon, 1998). Lastly, nitrogenase gene expression is controlled

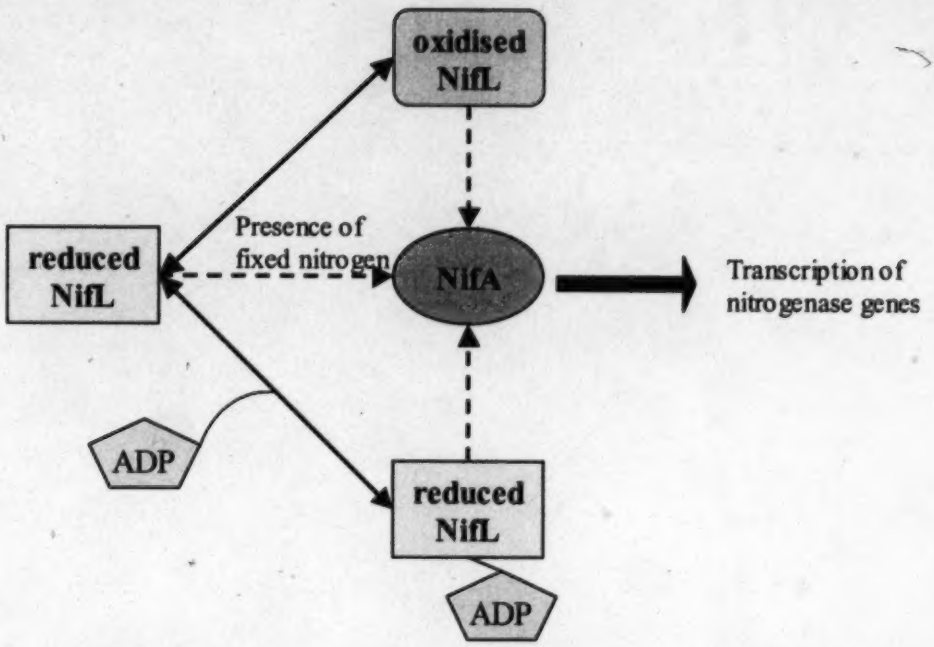

Figure 1.1 Regulation of nitrogenase gene expression by the NifL and NifA two-component regulatory system. Conversion of inactive NifL to forms that are inhibitory to NifA (indicated by the dashed arrows). Oxidized NifL inhibits NifA activity. The reduced ADP bound form of NifL is also inhibitory to NifA activity. NifL is also able to sense the presence of fixed nitrogen will inhibit NifA activity when there is excess ammonia (see text for details). Adapted from Dixon (1998).

by the presence of fixed nitrogen. The mechanism by which NifL senses the levels of fixed nitrogen are less well understood but it does involve interaction with GlnK, a component of the global nitrogen-sensing cascade (Little *et al.* 2002).

One of the biggest challenges encountered by nitrogen-fixing organisms is the oxygen sensitivity of nitrogenase. Nitrogenase becomes irreversibly inactivated by oxygen likely due to the action of O_2 and other oxygen radical species on the metal centres of the enzyme (Robson & Postgate, 1980). For obligate aerobic nitrogen-fixing bacteria like *Azotobacter* spp., several mechanisms are used to protect the nitrogenases. One possible mechanism is the long held hypothesis of respiratory protection. This hypothesis suggests that high respiratory activity of cells removes oxygen at the cell surface, and thereby prevents the oxygen from entering the cells and destroying the nitrogenase (Robson & Postgate, 1980). Respiratory protection is a problematic model for several reasons. For one, it has not been proven that the high respiratory rate of Azotobacter species at high ambient oxygen concentrations prevents oxygen from entering the cells. Second, the high respiratory rate of *A. vinelandii* is due to the activities of the terminal oxidase cytochrome *bd* complex, but the regulation of the genes that encode this complex is not dependent solely upon oxygen concentration, but also upon carbon-nitrogen ratios in the cell (Oelze, 2000). Another mechanism that *A. vinelandii* uses to protect nitrogenases is conformational protection. Conformational protection is mediated by the 24 kDa Shethna protein, a dimer with two 2Fe-2S centres. This protein will reversibly bind to nitrogenase and protect it from oxygen damage (Robson & Postgate, 1980). Recently Sabra *et al.* (2000) have implicated the extracellular polymer alginate in the protection of nitrogenase from oxygen. The amount

and structure of alginate is altered during growth in high oxygen tensions. In high oxygen tensions the alginate tends to be more thick and compact compared with alginate formed at lower oxygen tensions. Sabra *et al.* (2000) have suggested that the thick and compact alginate capsule acts as an oxygen diffusion barrier and therefore protects nitrogenase.

1.2.3 Cyst formation, poly-β-hydroxybutyrate, and alginate production

One of the strategies which *A. vinelandii* uses to deal with environmental stress is the formation of metabolically quiescent and desiccation-resistant cysts. The process of encystment is a systematic process in which cells begin accumulating poly-β-hydroxybutyrate (PHB), and terminate DNA replication, nitrogen fixation, and cell division. Mature cysts are spherical and have a multi-layered coat that contains a high proportion of alginate (Sadoff, 1975). Cysts may lay dormant in soils for up to ten years and are resistant to a wide variety of physical parameters although they do not exhibit the remarkable heat resistance observed in bacterial endospores. Germination and outgrowth of cysts is a sequential set of activities, which includes respiration followed by RNA and protein synthesis and later DNA synthesis. The cells emerge from the outer cyst coat and elongate, and will regain their flagella before they divide (Sadoff, 1975).

The two polymers PHB and alginate, in addition to being essential for encystment, both have industrial importance. PHB is an intercellular polymer that is produced by *A. vinelandii* as a carbon and energy storage compound. Many different species of bacteria are able to form PHB and its uses as a biodegradable thermoplastic are

currently being explored (Page *et al.*, 2001). In contrast to PHB, bacterial alginate is an extracellular polymer produced by relatively few prokaryotes. It is a polymer of unbranched β-D-mannuronic acid and α-L-guluronic acid; the composition of alginate varies among different strains. This variation is caused by the action of epimerises, which catalyze the conversion of mannuronic acid to guluronic acid. *A. vinelandii* has six such epimerises involved in this process (Hilde *et al.*, 2000). Upon addition of calcium, alginate will form a heat resistant gel that has potential application in many industries (Sabra *et al.*, 2001).

1.2.4 Electron transport chain

As mentioned previously the respiratory rate of *A. vinelandii* is the highest observed for any organism (Robson & Postgate, 1980). It is an obligate aerobe and therefore only able to use oxygen as a terminal electron acceptor. Like many other prokaryotes *A. vinelandii* possesses a branched electron transport chain. The overall structure of the electron transport chain as it is currently understood is shown in Figure 1.2. Electrons are supplied to the transport chain via several different dehydrogenases. There are two NADH ubiquinone oxidoreductases, NDH I and NDH II (Bertsova *et al.*, 1998). The NDH I is coupled to proton translocation across the membrane to form a Δp. This complex extrudes two protons for every electron ($H^+/e^- = 2$). The NDH II complex is not coupled to proton translocation and it is unregulated during diazotrophic growth at high oxygen tensions (Bertsova *et al.*, 1998; Bertsova *et al.*, 2001). NDH II-deficient mutants are unable to grow in nitrogen-limiting high aeration conditions, but they are

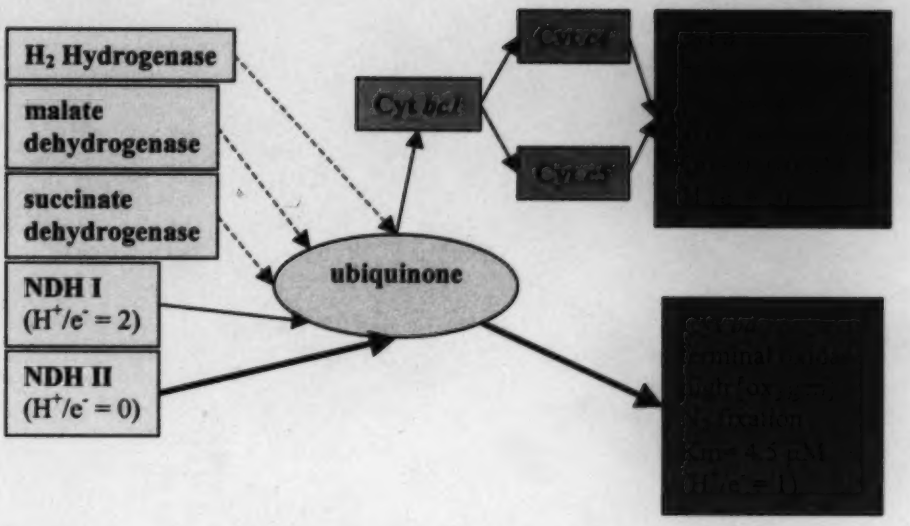

Figure 1.2 Diagram of the branched electron transport chain of *A. vinelandii*. Cyt, cytochrome; NDH, NADH ubiquinone oxidoreductase. The proton(s) translocated across the membrane per electron passed through the complex are shown. Thick arrows indicate the low energy-yielding pathway, thin arrows show the high energy-yielding pathway. The dashed arrows indicate electron sources that can feed into either high or low energy-yielding pathways. Adapted from (Oelze, 2000) and (Bertsova *et al.* 2001).

fully capable of growth in low aeration when supplied with NH_4^+ (Bertsova *et al.*, 2001) Another source of electrons is the hydrogenase enzyme. This complex is capable of using hydrogen, a by-product of nitrogen fixation, and donating its electrons to the electron transport chain (Robson & Postgate, 1980). In this way *A. vinelandii* is able to cope with a potentially wasteful product and derive some energy from it. Other important electron donors include malate and succinate dehydrogenases from the citric acid cycle (Oelze, 2000).

The electrons are shuttled to a ubiquinone carrier before they are passed on to one of two terminal oxidases. One of these branches leads to a cytochrome *bc1* complex that feeds into either cytochrome *c4* or *c5* (Ng *et al.*, 1995). Both of these cytochromes feed their electrons to a cytochrome *o* type oxidase. This terminal oxidase has a high affinity for oxygen with a Km value of approximately 0.016 μM (D'Mello *et al.*, 1994). In this pathway there are three protons pumped for every electron ($H^+/e^- = 3$) (Bertsova *et al.*, 1997). The second terminal oxidase branch is much simpler and consists of a single cytochrome *bd* type quinol oxidase. This complex has a much lower affinity for oxygen with a Km of 4.5 μM (D'Mello *et al.*, 1994) and it only translocates one proton for every electron ($H^+/e^- = 1$) (Bertsova *et al.*, 1997). The regulation of these components is very complex and it is currently believed that the high energy yielding branch (Figure 1.2, thin arrows) is upregulated during ammonium assimilation and low oxygen concentrations whereas the lower energy yielding branch (Figure 1.2, thick arrows) is important for diazotrophic growth at high oxygen concentrations (Bertsova *et al.*, 2001). Mutants in the cytochrome *bd* terminal oxidase pathway are impaired in their ability to fix nitrogen at high oxygen concentrations (Kelly *et al.*, 1990). However ambient oxygen

concentration alone does not control the composition of the electron transport chain in *A. vinelandii*, rather it is highly dependent upon carbon and nitrogen availability especially at ambient oxygen concentrations of 70 μM and higher (Oelze, 2000).

1.2.5 *Azotobacter* motility

In his initial descriptions of *A. vinelandii* in 1903, J. Lipman describes the cells as possessing a "progressive and at times rotary motility" (as cited by Thompson & Skerman, 1979). The only other descriptions of *A. vinelandii* motility for the better part of a century concerned the arrangement of flagella, and the loss and acquisition of flagella during encystment and germination, respectively. Not all *Azotobacter* species are motile, as *Azotobacter beijerinckii* and *Azotobacter nigricans* are aflagellate and non-motile. *Azotobacter chroococcum*, *Azotobacter armeniacus*, *Azotobacter salinestris* and *Azotobacter paspali* like *A. vinelandii* are all motile by means of peritrichous flagella (Kennedy *et al.*, 2001). The first systematic study of *Azotobacter* motility was reported by Haneline *et al.* (1991). In that study the tactic response of *A. vinelandii* to a variety of carbohydrate compounds was investigated. It was found that a variety of compounds were chemoattractants including: glucose, gluconate, glycerol, sucrose, fructose, ribose, arabinose and mannose. All of these compounds induce a smooth swimming behaviour characteristic of chemoattractants (see Section 1.3.2). Galactose and xylose, on the other hand, were repellents and caused the cells to tumble. Some of these results are surprising as ribose, arabinose, and mannose do not support *A. vinelandii* growth as sole carbon sources; conversely the repellent galactose is able to support growth, but xylose is not

(Thompson & Skerman, 1979). Analogs of glucose such as α-methyl-D-glucoside did not act as chemoattractants (Haneline *et al.*, 1991). Based on time-lapse film exposure, the researchers were able to estimate the swimming speed of *A. vinelandii* cells in liquid at 74 μm/s. Interestingly, cells could be induced for taxis towards certain compounds. For example cells pre-grown on glucose and fructose responded to glucose and fructose, respectively, but were not able to respond to mannitol or xylitol. Similarly mannitol pre-grown cells responded to both mannitol and xylitol. This metabolism-dependent response is characteristic of energy taxis. One notable omission by Haneline *et al.* (1991) was the lack of description of taxis toward organic acids and compounds of the tricarboxylic acid cycle, since it had previously been shown that these were preferred growth substrates for *A. vinelandii* (George *et al.*, 1985).

1.3 Bacterial motility

1.3.1 History

The study of bacterial motility dates back to the very roots of microbiology (for an excellent review on the history of bacterial motility see Armitage, 1997) It was the deliberate swimming movement of particles in pepper water that in 1676 led Dutch microbiologist Antony van Leeuwenhoek to the conclusion that he was observing living creatures. These "wee animalcules", as Leeuwenhoek called them were almost certainly bacteria as he described them as being a thousand-fold smaller than the other specimens he had previously observed (Armitage, 1997). His observations were published in a

series of letters to the Royal Society of London in which he described in remarkable detail the motile behaviour of his specimens. Upon addition of vinegar to the samples the cells stopped moving and from this Leeuwenhoek deduced that the cells were dead. Motility was Leeuwenhoek's primary criterion for life. Over the years he recorded numerous observations of motile bacteria isolated from a wide variety of environments. When looking at what must have been protozoa, he could see their flagella and cilia, which he described as "paws". For the "wee animalcules" he could see no similar structures, yet he speculated that these creatures were equipped with related appendages (Armitage, 1997). Leeuwenhoek's most famous observations were written in a letter to the Royal Society of London in 1683 in which he drew a figure of five different species that he collected from his own tooth scrapings. He described: "an unbelievably great number of living animalcules, swimming more nimbly than any I have seen up to this time. They bent their bodies in such curves going forward-they were in such number that all the water seemed to be alive" (Armitage, 1997).

It was not until the late nineteenth century that further descriptions of bacterial motility were made. The German scientist Thomas W. Engelmann provided the earliest scientific studies on bacterial behaviour towards light and oxygen. In 1881 Engelmann first described aerotaxis, the motile response of bacteria towards oxygen; he observed that the microaerophile *Spirillum tenue* was attracted to low oxygen concentrations and repelled by high oxygen concentrations. Subsequent to this, Martinus Beijerinck went on to describe how motile species of bacteria, when placed in an oxygen gradient, would form a distinct band. The position of this band was species-dependent, and Beijerinick called this band "*atmungsfiguren*" (Taylor & Zhulin, 1999). Coincidently in 1901

Beijernick became the first microbiologist to isolate and describe a species of *Azotobacter* (Thompson & Skerman, 1979).

By the end of the nineteenth century a great deal of information on the motile behaviour of bacteria had been gathered. Despite this, there was little insight gained into the mechanisms behind taxis responses until the groundbreaking work of Julius Adler in the 1960's. He used soft agar swarm plates to investigate the chemotactic ability of bacteria towards different carbon substrates (Adler, 1965; Armstrong *et al.*, 1967). These soft agar plate assays relied on the bacteria to metabolize energy sources within the media and thus create their own natural gradient with which to respond chemotactically. These elegant experiments led to the important discoveries in the mechanisms behind bacterial sensing, signalling and taxis. We will now look at the taxis pathway in *Escherichia coli*, as it is currently understood; keeping in mind that *E. coli* has the most well characterized taxis pathway of any prokaryotic organism. If we can gather anything from the historical accounts of bacterial motility, it is that there is incredible diversity in the natural world, and no one organism is representative of all species.

1.3.2 The taxis pathway in *E. coli*

The taxis pathway in *E. coli* has been extensively studied (for reviews see: Armitage & Schmitt, 1997; Bren & Eisenbach, 2000; Stock & Surette, 1996; Taylor *et al.*, 1999). The term "chemotaxis" has been used to describe the response of cells to chemical attractants or repellents. In his early experiments with *E. coli*, Adler discovered that response towards a certain compound was unaffected in mutants unable to transport

or metabolize that compound (Adler, 1969; Ordal & Adler, 1974). These and subsequent experiments confirmed that there were specific chemoreceptors in *E. coli* that governed taxis. The exceptions to this trend were the sugars of the phosphotransferase system (PTS) which required transport into the cell in order to elicit a taxis response (Adler & Epstein, 1974). The phenomenon of aerotaxis or energy taxis is also modulated by specific receptors. The nature of the receptors for aerotaxis and energy taxis will be discussed in Sections 1.4 and 1.5. All forms of taxis (chemotaxis, PTS-taxis, aerotaxis, and energy taxis) characterised in *E. coli* use a common signalling system, the Che system (Rowsell *et al.*, 1995; Taylor *et al.*, 1999)

There are six Che proteins in *E. coli* that are involved in taxis signal transduction CheA, CheW, CheY, CheZ, CheB, and CheR. There are two important multi-protein signalling complexes in the taxis response. The first complex is the taxis transducer protein-CheA-CheW ternary complex that is involved in sensing tactic stimuli. Taxis transducers are membrane-associated proteins that are involved in sensing the status of their environment and relaying this information to the Che proteins. These taxis transducer proteins are a remarkable superfamily of proteins whose structure and function will be discussed in section 1.4. The second important multi-protein complex is the flagellar-motor complex randomly distributed around the cell and distributed in the cell membrane. CheY is the protein messenger that shuttles between these two complexes and transduces the input signal from the transducer proteins to the flagellar motor complexes. The signal transduction is mediated by a series of protein phosphorylation events and protein-protein interactions; the entire scheme is presented in Figure 1.3.

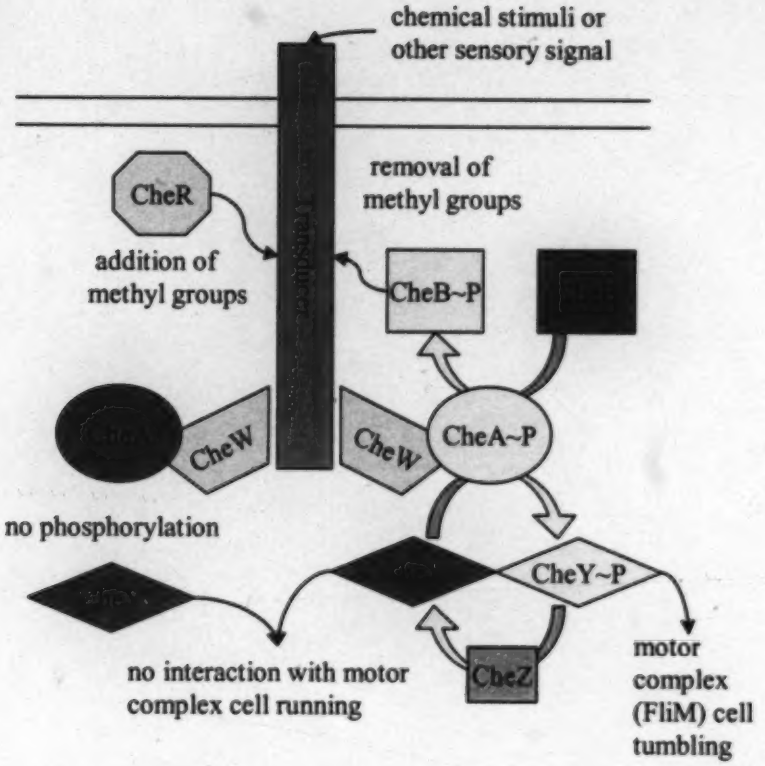

Figure 1.3 Schematic chemosensory pathway in *E. coli*. Transducer proteins are in a ternary complex with CheW and the sensor kinase CheA. Upon sensing a negative gradient of attractant (*i.e.* the transducers sense attractants temporally and they sense less at one moment than they did seconds earlier) CheA will be signaled to autophosphorylate and will become CheA~P. CheA~P will pass its phosphoryl group onto two response regulators, CheY and CheB. CheY~P interacts with the FliM protein at the flagellar motor and results in CW rotation and cell tumbling. Positive gradients of attractant result in no phosphorylation of CheA or CheY and flagella rotate CCW and the cell continues swimming. CheR and CheB are involved in adaptation to stimuli (see text for details).

CheA is a sensor kinase that is a member of the two-component signal transduction family. It autophosphorylates at a conserved histidine residue using Mg-ATP as a phosphodonor (Stock & Surette, 1996). The associated taxis transducer protein regulates the autophosphorylation activity of CheA both positively and negatively. The linker protein, CheW, is critical for CheA phosphorylation, and for CheA interaction with the transducer. CheW increases the affinity of CheA for ATP by nearly 70-fold (Amsler & Matsumura, 1995), thereby upregulating CheA autophosphorylation. CheW appears to have independent binding sites for CheA and the taxis transducer protein, and may facilitate signalling from the transducer to CheA (Stock, 1996). Once CheA is phosphorylated it transfers its phosphate group to its cognate response regulator CheY. Phosphorylated CheY (CheY~P) interacts with the flagellar motor complex and switches the motor rotation from the default counter-clockwise (CCW) to clockwise (CW) direction (Eisenbach, 1996). *E. coli* is a peritrichous bacterium with an average of six flagella, and when the flagella are rotating CCW they form a bundle and the cells swim smoothly. Conversely when the flagella are rotating CW the flagella rush apart, and the cells tumble. This change of swimming to tumbling behaviour serves to randomly reorient the cell allowing it to swim in a new direction as soon as it gets a signal to resume CCW rotation of the flagella. The temporal length of the swims and the tumbles is ultimately determined by the transducer proteins and will bias the cells direction to a more favourable environment. The longer the swims indicate the transducers are sensing an attractant. Conversely the more frequent the tumbles indicate the cells are moving away from attractants or sensing a repellent (Armitage & Schmitt, 1997).

The proportion of phosphorylated CheY in the cell is what directly determines whether or not the cells will swim or tumble. The more CheY~P, the longer the cells will tumble and conversely the more CheY, the longer the cells will swim. After a period of tumbling, a cell is no longer in the same orientation and therefore when they receive a CCW signal they begin to swim in a new direction. When cells are in an environment where there is a gradient of attractant they suppress their tendency to accumulate CheY~P and the cells swim smoothly (Blair, 1995). A repellent has the opposite effect, in that it causes the cells to tumble. Equally, when the cells are in a homogeneous environment they switch between tumbling and swimming at a constant rate evidenced by the so-called random walk. Cells can also become adapted to their environment and in doing so will no longer respond to attractants. Adaptation involves the methylation of taxis transducer proteins and the proteins CheB and CheR.

If there is an attractant present, the taxis transducer protein will send a signal to CheA not to phosphorylate. Since CheA is not phosphorylated, CheY will not become phosphorylated and the cell will swim. If however there is no attractant present or there is a repellent effect then CheA will autophosphorylate, at the conserved histidine residue, and transfer its phosphate group to CheY. The autophosphorylation of CheA is believed to occur when CheA is in a dimeric form. Like most response regulators, CheY contains the catalytic residues required for its own phosphorylation (Stock & Surette, 1996). It has been shown that CheY can catalyze its own phosphorylation using low molecular weight phosphodonors, such as acetyl phosphate, instead of CheA (Lukat *et al.*, 1992). However, the importance of this activity *in vivo* is disputable as CheA-mediated

phosphorylation occurs at much higher rates than acetyl phosphate-mediated phosphorylation (Mayover *et al.*, 1999).

The dephosphorylation of Che-Y~P is hastened by the protein CheZ. Mutants lacking *cheZ* tumble constantly. When CheY~P is bound to the flagellar motor complex it is protected from dephosphorylation (Bren *et al.*, 1996). As CheY~P is dephosphorylated the proportion of free CheY~P in the cell changes. This shifts the equilibrium of flagellar motor complex bound CheY~P versus free CheY~P. The result is a decrease in flagella rotating CW. Only phosphorylated CheY will bind CheZ, this is achieved through oligomerization of native CheZ dimers (Blat *et al.*, 1998). After the two proteins are bound there is a 50-100 ms delay before CheY~P is dephosphorylated (Bren & Eisenbach, 2000). It is believed that this delay serves to ensure that the tumbling behaviour of the cells is not halted prematurely (Bren & Eisenbach, 2000). The catalytic phosphatase activity is found on the CheY protein and CheZ serves to realign CheY~P into its active phosphatase conformation (Stock & Surette, 1996).

E. coli has five known taxis transducer proteins: Trg, Tsr, Tap, Trg, and Aer. The first four taxis transducer proteins have conserved glutamate residues that are methylated by the protein CheR. Tar, Tap, Trg, and Tsr were the first taxis transducer proteins discovered and they were termed methyl-accepting chemotaxis proteins, (MCP's) because methylation was found to be an important feature of these proteins. There are three to four methylation targets on each these transducers. If one of these transducers is fully methylated the autophosphorylation of CheA increases nearly 100-fold. This ultimately results in the transmission of a CW signal and cell tumbling (Stock & Surette, 1996). Li and Weis (2000) have shown that covalent binding of methyl groups to

transducers regulates ligand binding in the sensing domains. The result of this methylation is that the transducer-CheW-CheA ternary complex will no longer respond to attractants. Therefore if a cell has been in an attractant-rich environment long enough it will no longer swim smoothly but will tumble. The cell is rescued from this tumbling behaviour by the protein CheB. CheB is another response regulator whose function is to remove methyl groups from the transducer. The cognate sensor kinase of CheB is CheA. Both CheY and CheB have comparable affinities for CheA and therefore tend to compete for CheA phosphorylation (Li *et al.*, 1995). CheB~P is a methylesterase that catalyses the removal of methyl groups from the transducer to form methanol (Amsler & Matsumura, 1995; Stock & Surette, 1996). Once the transducers are demethylated they can again respond to attractants.

The taxis response in *E. coli* is a complex system. It allows the cells to efficiently and rapidly respond to environmental stimuli without altering gene expression. *E. coli* is cited extensively in taxis studies and the chemotaxis system in this organism is arguably the best characterized signal transduction system in nature. Although *E. coli* is a model organism for taxis responses it is not representative of the diversity of motility and taxis found in other prokaryotes. The goal of the next section is to introduce taxis responses in non-enteric organisms in order to broaden our understanding of the phenomenon of bacterial motility.

1.3.3 Taxis responses in other bacteria

Most motile bacteria move with a similar punctuated response in order to move towards or away from stimuli. When faced with a gradient of attractant or repellent, cells swim longer in the presence of attractant or absence of repellent, thereby biasing their overall movement towards an optimal environment. However, unlike *E. coli*, many bacteria are unable to rotate their flagella in two directions to swim and tumble. For example, *Rhodobacter sphaeroides*, a photosynthetic non-sulphur purple bacterium, has a single lateral flagellum that only rotates in one direction. The flagellar motor in *R. sphaeroides* changes speed or stops periodically, enabling the cells to move in a 3-dimensional swimming pattern (Armitage & Schmitt, 1997). The symbiotic nitrogen fixing bacterium *Sinorhizobium meliloti*, possesses a bundle of flagella which only rotate CW; but the speed of the rotation can change, thereby changing the direction of swimming (Armitage & Schmitt, 1997).

The Che signal transduction system that governs motility in *E. coli* appears to be an ancient system that is found in all motile prokaryotes that have been examined. Homologues of *cheA*, *cheW* and *cheY* have been found in archaea and eubacteria (Bourret *et al.*, 2002). So far, the genomic sequences of all motile organisms contain homologues of *cheB* and *cheR*, with the notable exception of *Helicobacter pylori* (Bourret *et al.*, 2002).

One of the most impressive characteristics of prokaryotic organisms is the vast metabolic diversity possessed by different species. It is only appropriate that in such a diverse group there are numerous stimuli that elicit tactic responses. Some of the better-known tactic responses include aerotaxis, energy taxis, magnetotaxis, phototaxis, and chemotaxis. Aerotaxis is defined as the movement of cells in response to oxygen.

Similarly energy taxis is defined as the movement of cells in response to stimuli that affect intracellular energy levels. Magnetotaxis is the remarkable behaviour of some bacteria characterised by movement along geomagnetic lines. This movement is governed by specific organelles in the bacterial membranes known as magnetosomes. Magnetosomes are not responsible for signalling a magnetotactic response *per se*, but instead use the geomagnetic force to align the bacteria along the earth's magnetic field. Most magnetotactic bacteria are microaerophiles and they use magnetotaxis in conjunction with aerotaxis to align themselves in a specific microaerophilic environment (Schuler & Frankel, 1999). Phototaxis, the response to light, is another behavioural response that has been known for well over a century. The mechanics behind phototaxis are well studied in the archeon *Halobacterium salinarium* and in the eubacterium *R. sphaeroides*. Lastly, chemotaxis is the behavioural response of bacteria to chemicals. The chemicals that serve as attractants and repellents vary from species to species and appear to depend on the physiology and lifestyle of the species. All of these responses, with the exception of magnetotaxis, are mediated by a superfamily of proteins known as taxis transducer proteins. The structure, function, and characteristics of these proteins will be discussed next.

1.4 The Superfamily of taxis transducer proteins

1.4.1 Salient features of transducer proteins

Earlier in this work there was discussion of taxis transducer proteins and their role in directing motility. In this section we will take an in depth look at this superfamily of proteins. A note on terminology; for historical reasons these proteins have been called MCP's or chemoreceptors. This is largely due to J. Adler's work on chemotaxis in *E. coli* and the observation that the transducers are methylated (Armitage, 1997). However not all transducers are methylated, nor are they all involved in chemotaxis *per se,* but maybe involved in other tactic responses. Therefore in order to avoid confusion throughout this work these proteins will be referred to either as taxis transducer proteins or simply as transducers.

Taxis transducer proteins are found exclusively in motile prokaryotes, although many of the domains within these proteins are founds in eukaryotes (Zhulin, 2001). For nearly thirty years there were only four known transducer proteins in *E. coli*: Tar, Tap, Trg, and Tsr, referred to here as the classical transducers. Not until the *E. coli* genome was sequenced did researchers discover a fifth transducer protein Aer (Bibikov *et al.*, 1997; Rebbapragada *et al.*, 1997) responsible for mediating taxis towards oxygen and energy. The elusiveness of Aer is remarkable considering that taxis in *E. coli* is one of the best characterized signal transduction schemes in microbiology. There are already more than a thousand putative sequences of transducer proteins available on public databases (Alexandre *et al.*, 2004) with further sequencing of microbial genomes it is believed that hundreds more transducer proteins will be identified in a wide variety of species (Zhulin, 2001).

Not all transducers are distributed equally within the cell membrane, remarkably they tend to aggregate near the poles of the cell (Alley *et al.*, 1992; Maddock & Shapiro,

1993),. The functional role that this clustering may play is not well understood however, CheW has been implicated in polar localization of transducers in *E. coli* (Skidmore *et al.*, 2000). Similar polar localization has been observed in *R. sphaeroides* (Harrison *et al.*, 1999) and *Caulobacter cresentus* (Alley *et al.*, 1992) suggesting that this could be a widespread characteristic of transducers. Recently it has been suggested that this clustering of transducers in *E. coli* allows for integration of signals among different transducers and amplification of subtle signals (Sourjik & Berg, 2004)

Transducers are found as homodimers within the cell (Mowbray & Sandgren, 1998; Stock & Surette, 1996). It is believed that this dimerization is necessary for signal propagation within the transducer-CheW-CheA ternary complex. However, much higher oligomers of transducers have been identified (Li & Weis, 2000; Liu *et al.*, 1997). The crystallized transducer Tsr of *E. coli* exists as a trimer of dimers which are joined at the distal portion of the cytoplasmic domain involved in CheA and CheW interactions (Kim *et al.*, 1999). Computational studies suggest that oligomerization may result in increased sensitivity for detecting tactic stimuli, although the concentration range of compounds that would be detected would be limiting (Bray *et al.*, 1998). There is increasing evidence that the assembly and disassembly of transducer-CheW-CheA ternary complexes in the membrane is a dynamic process, and that oligomerization of transducer homodimers is common for signal propagation in taxis responses (Bren & Eisenbach, 2000; Li & Weis, 2000).

1.4.2 Domain organisation and the different classes of transducer proteins

The best-studied transducers to date are the classical transducers in *E. coli*. Discussions on domain organisation and sequence features will largely involve these four proteins. However whenever possible other examples will be included to reflect the diversity of this superfamily of proteins. Domain organisation and primary structure of the classical transducers as in *E. coli* and *Salmonella typhimurium* are shown in Figure 1.4 (Class I). The proteins all have a short tail that extends into the cytoplasm followed by the first of two transmembrane domains (TM1). The variable sensory domain is situated in the periplasm, followed by the second transmembrane domain (TM2). A linker domain connects the TM2 domain to the first methylated helix domain (MH1), the signalling domain, also known as the highly conserved domain (HCD), and finally the second methylated helix (MH2) (Stock & Surette, 1996).

The signature element of the superfamily of taxis transducer proteins is the highly conserved domain (HCD). It is the signalling domain that interacts with CheW, and allows for signal transduction with the Che proteins. Surette & Stock (1996) demonstrated this binding by expressing the HCD from *tar* of *E. coli*; Tar folded independently *in vivo* and was able to bind CheW. Genetic studies have also implicated the HCD in CheW binding (Liu & Parkinson, 1991). Le Moual and Koshland (1996) compared the C-terminal sequence of 29 transducers from 16 species, including gram-positive and gram-negative bacteria and archaea. They were able to derive a consensus secondary structure for the HCD. Since that time the sequences of many other transducer proteins have become available. Zhulin (2001) has updated the work of Le Moual & Koshland (1996) and has generated a 90% consensus for the HCD through the multiple alignment of more than 120 transducers. A distinct hairpin loop and an α-helical

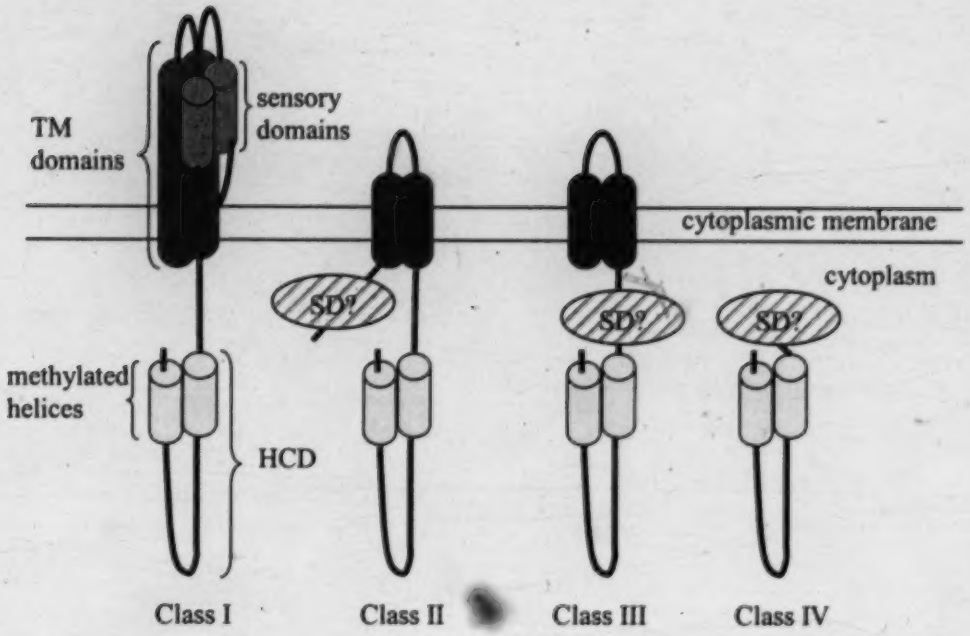

Figure 1.4 Topological classes of taxis transducer proteins. All transducers are shown as monomers. TM domains are shown as dark cylinders that span the cytoplasmic membrane. Class I transducers include: Tar, Tsr, Trg and Tap of *E. coli*. The sensory domains in Class I transducers separates the two TM domains. Class II transducers, such as Aer in *E. coli*, contain a longer N-terminal region that may encode a sensory domain (SD). Class III transducers contain two adjacent TM domains at the N-terminal segment of the protein. Sensing in these transducers is believed to occur between the TM2 and MH1 domains. Class IV transducers encode a HCD but have no predicted TM domains and are believed to be cytoplasmic. The sensory domains in Class II, III, and IV transducers are variable, as indicated by the hatched ovals. Adapted from Zhulin (2001)

structure are characteristic of the HCD. This consensus of HCD allows for straightforward identification of transducer proteins in sequence databases. Expectation values (e-values) computed by a BLAST search for most HCD domains are very low, 10^{-50}, indicating significant conservation among these domains.

The MH1 and MH2 domains are sites for methylation by the CheR methyltransferase. Experimental evidence as well as computational analysis suggests that these regions are likely to form α-helical coiled coils (reviewed in: Mowbray & Sandgren, 1998; Stock & Surette, 1996). Methylation occurs at specific glutamate residues that can then be demethylated by the response regulator CheB~P. In some cases the methylated residues were originally glutamine residues that were deaminated by CheB~P, which possesses an amidase activity in addition to its methylesterase activity. The number of residues that are subject to methylation varies from transducer to transducer. In their study on transducer homology (Le Moual & Koshland, 1996) identified potential methylation sites on most of the transducers they studied, including those from bacteria and archaea. However several transducers including two from *Vibrio cholerae* and one from *Desulfovibrio vulgaris* had sequences so variable that it was questionable whether they are actually methylated (Le Moual & Koshland, 1996).

Methylation of transducer proteins is not as universal as previously alleged. For example a transducer in *S. meliloti* has been experimentally shown not to methylate (Greck *et al.*, 1995). In *Bacillus subtilis* adaptation to attractants and repellents is believed to occur through both methylation-dependent and methylation-independent methods (Kirsch *et al.*, 1993a; Kirsch *et al.*, 1993b). The selective methylation of particular sites in the McpB transducer of *B. subtilis* is another strategy this organism

uses to adaptation its taxis response to its environment (Zimmer *et al.*, 2000). The pattern of methylation and demethylation on transducers in other species such as *H. salinarium* and *R. sphaeroides* has also been the subject of investigation (Zhulin, 2001). Transducers in these organisms demethylate in response to both attractants and repellents. More research is needed to understand the nature of methylation on different transducer proteins, especially in non-enteric systems.

The linker domain connects the MH1 with TM2. Recently this domain has been reclassified as the HAMP domain (Zhulin, 2001). The term HAMP comes from the three different families of proteins in which this domain is found: *h*istidine kinases, *a*denylate cyclases, and *m*ethyl-accepting chemotaxis *p*roteins (Aravind & Ponting, 1999). It is believed that this region of the transducer is important for transmitting the signal from the N-terminus to the C-terminus of the protein via a conformational change.

Transducers have two α-helices spanning the membrane: TM1 and TM2. Studies have shown that the transduction of a signal through the transmembrane domains is mediated by pivoting the TM2 domains relative to the central axis of the dimer (Mowbray & Sandgren, 1998). Studies on the Tar transducer of *E. coli* show that the protein is still capable of ligand binding and signalling *in vitro* even when the transmembrane domains have been removed (Ottemann & Koshland, 1997).

There are four different topological classes of transducer proteins. The basic structures of these classes are given in Figure 1.4. In his phylogenetic study of transducer proteins (Zhulin, 2001) used computational analysis to predict the topology of transducer from a variety of genera. The structural topology of a transducer is not a predictor of function. For example, Aer in *E. coli* and YoaH of *B. subtilis* are class II transducers but

their N-terminal sensing domains are very different (Bibikov *et al.*, 2000; Zhulin, 2001). On the other hand, both these transducers have intracellular sensing domains, suggesting some sort of internal monitoring behaviour. The function of Aer has been characterised experimentally, while the function of YoaH is still not known. The classical transducers of *E. coli* are all class I transducers, these proteins contain a large periplasmic or extracellular domain tethered to the membrane via two transmembrane domains. Class III transducers also contain intracellular sensing domains, and are anchored into the membrane via the N-terminal. Two examples of these proteins are CetA of *Campylobacter jejuni*, involved in energy taxis (Hendrixson *et al.*, 2001), and a putative photoreceptor in *Synechocystis* spp. (Zhulin, 2001). Class IV transducers contain no TM domains and are believed to be cytoplasmic (Figure 1.4). These transducers may be one part of a bipartite system, or they may function independently; further investigation into class IV transducers is needed.

In some of the classical transducers, periplasmic binding proteins are required in order for the attractant to bind to the transducer (Stock & Surette, 1996). These include: maltose-binding protein, ribose binding protein and dipeptide binding protein. Some transducers respond to multiple signals. Such is the case for the serine transducer Tsr that has been implicated in mediating behaviour to: serine, alanine, glycine, external pH, temperature, hydrophobic amino acids, indole, and most recently energy (Rebbapragada *et al.*, 1997; Stock, 1997). The multifunctional nature of these proteins may be a strategy that allows the cell to sense a variety of stimuli with a minimal amount of machinery. This also poses a challenge for the experimental characterization of the substrate specificity of transducer proteins.

The sensing domains of transducers are highly variable and reflect the diversity within the superfamily. In the classical transducers the overall structure of the periplasmic domain is very similar, consisting of four α-helical bundles with two ligand binding sites (Mowbray & Sandgren, 1998). There are three other conserved sensing domains found in transducer proteins, one of which is the PAS domain, involved in oxygen and energy sensing (Taylor *et al.*, 2001). The physiological function of PAS domains is governed by bound co-factors such as flavin molecules or heme groups. An in depth review of PAS domains will be discussed in Section 1.5.3. The GAF domain is another domain that has been identified in transducers, and it is implicated in light dependent signal transduction (Zhulin, 2001). GAF domains are found in eukaryotes, eubacteria, and archaea; they contain conserved residues that appear to bind tetrapyrrole chromophores, which are likely responsible for the light sensing ability of these proteins. The ESENS domain, which was named for its role in *e*xtracellular *sens*ing, are found in a wide variety of proteins including histidine kinases and adenylate cyclases, and are believed to sense small molecules such as amino acids (Zhulin, 2001). Class I transducers with ESENS domains have been found in *B. subtilis*, *Pseudomonas aeruginosa*, and some archaea. A nitrate sensing domain, NIT, has also been identified in several transducers (Shu *et al.*, 2003). Another conserved domain of unknown function, the CHASE domain, has been identified in several transducers and in other signal transduction proteins (Zhulin *et al.*, 2003).

The key features of this family of proteins are their remarkable diversity coupled with high conservation. The key point is that genetic sequence can only identify a putative function of a transducer, while biochemical and physiological investigations are

required to fully understand how transducers work. Currently there is an enormous gap in our knowledge of how these transducers function, especially in non-enteric systems. The sequence data of transducers have exploded, but these are accompanied by relatively few experiments to explore the nature and function of this fascinating superfamily of proteins.

1.4.3 Diversity and distribution of taxis transducer proteins

One of the first studies to investigate the distribution of transducers in nature was carried out by Morgan *et al.* (1993). In this study the researchers used an anti-Trg-HCD antibody in various Western blots of total cell protein from a variety of genera. They found that the antibody reacted with different bacteria and at least one archeon, suggesting that transducers are widespread. More recent phylogenetic analysis by Alexandre *et al.* (2004) identify transducer proteins in over 120 species of motile prokaryotes. To date there are no examples of taxis transducer proteins in eukaryotes, although several domains such as PAS, HAMP, GAF and ESENS found in transducers are also found in eukaryotic proteins, suggesting an ancient lineage. In all the microbial genomes that have been sequenced transducer genes accompany the presence of the che genes. Even in organisms where there is no flagellar motility, but gliding motility as in *Myxococcus xanthus,* transducers have been found. The number of transducer proteins per genome can vary from as few as two in *Archaeoglobus fulgidus* (Klenk *et al.*, 1997) to more than sixty in *Magnetospirillum magnetotactum* (Alexandre *et al.*, 2004). In some cases transducers are transcribed as part of a larger operon, but in many cases transducers

are located independently in a genome. For example, in *E. coli tar* and *tap* are part of what is called the meche operon, which also encodes for *cheR*, *cheB*, *cheY*, and *cheZ*. The other three transducers *tsr*, *trg*, and *aer* are encoded separately elsewhere in the *E. coli* genome. There are also examples of transducers found on plasmids (Yost *et al.*, 1998) and transposons (Allmeier *et al.*, 1992). Although there is no direct evidence of lateral gene transfer of transducer genetic sequences the possibility cannot be excluded. Taken as a whole, the diversity and distribution of taxis transducer proteins argues that this is an ancient superfamily of proteins that was present in a common bacterial-archael ancestor and is part of a widespread signal transduction system that modulates bacterial motility.

1.5 Aerotaxis and Energy taxis

1.5.1 Energy taxis as a survival strategy

One of the emerging features in bacterial motility is the notion that strict chemotaxis or strict aerotaxis may not be the principal behaviour that governs a cell to optimal microenvironments. Rather, energy taxis is increasingly looked to as the dominant behaviour in many bacteria. Energy taxis is a higher form of taxis that encompasses aerotaxis, chemotaxis, redox taxis and taxis to any number of other stimuli. Any of these stimuli will alter the flow of electrons (reducing equivalents) through the electron transport chain. The flow through the electron transport chain is directly proportional to the intracellular energy levels in the cell. The key feature of energy taxis

is the fact that it is a metabolism-dependent response (Taylor & Zhulin, 1998). A taxis transducer protein is responsible for sensing these changes in the electron transport chain, or possibly even the proton motive force, and subsequently directing a signal that tells the cells to swim or change direction (Taylor *et al.*, 1999).

Why is energy taxis a survival strategy for cells and not simply chemotaxis or aerotaxis? Imagine an environment where oxygen diffusion occurs along a very steep gradient. Strict chemotaxis may direct cells to a niche that is rich in carbon or nitrogen sources, but they may be in an oxygen-limiting environment. The cells would utilise the carbon or nitrogen source and in doing so respire at a high rate, quickly creating an anoxic environment. Energy taxis would lead the cells out of the growth-limiting anoxic environment, even though the cells would be moving away from a high concentration of nutrients. Similarly aerotaxis could direct cells to an optimal concentration of oxygen and lead the cells away from nutrients. Energy tactic behaviour would not direct cells to an oxic environment that is devoid of a carbon source and cannot maintain cellular energy. Furthermore strict aerotaxis may lead cells to an environment with a toxic level of oxygen. Energy taxis allows cells to quickly take stock of their intracellular energy levels and orient themselves appropriately. The phenomenon of energy taxis and aerotaxis in bacteria will be the focus of this section. In particular attention will be given to various transducers known to modulate these types of responses.

1.5.2 The aerotaxis transducer protein Aer in *E. coli*

Early studies of chemotaxis in *E. coli* clearly established the involvement of methylation in the motile response to a variety of chemicals. It was assumed that all tactic responses in *E. coli* would be methylation-dependent. Experiments by Niwano & Taylor (1982) demonstrated that aerotaxis and taxis to the sugars of the PTS system was unimpaired in *tsr*, *tar*, and *trg* mutants, however taxis to serine, aspartate and ribose was impaired. Subsequent experiments confirmed that methylation did not play a role in aerotaxis. The same research group went on to characterize the role of the Che proteins in aerotaxis. When *cheB* was mutated the cells had an inverted response to oxygen; that is to say, they were repelled by concentrations of oxygen that attracted wild type bacteria. When both *tsr* and *cheB* were mutated, the aerotactic response was comparable to wild type bacteria (Dang *et al.*, 1986). When *cheA*, *cheW* and *cheY* were mutated, aerotaxis was abolished, whereas genetic complementation or null mutants of these genes restored aerotaxis in *E. coli* (Rowsell *et al.*, 1995). These data together suggest that there is a unique transducer responsible for aerotaxis in *E. coli* that does not appear to be methylated by CheR, but does interact with the CheW, CheA and CheY proteins.

The nature of the signal for aerotaxis was also investigated. It was hypothesised that bacteria responding to oxygen concentrations would not sense oxygen *per se* but rather they would sense a change in their electron transport chain. Observations of aerotaxis over one hundred years ago support the notion that each species has a specific oxygen concentration to which it is attracted. *E. coli* displays taxis to the terminal electron acceptors nitrate, fumarate, and oxygen, however when the electron transport pathway to any of these compounds is impeded then taxis is abolished (Taylor *et al.*, 1979). Furthermore, a functional electron transport chain is necessary for the repellent

response to blue light (Taylor *et al.*, 1979). Shioi *et al.* (1988) demonstrated that taxis to oxygen was abolished in mutants of cytochrome *o* and cytochrome *d*, the terminal oxidases in the branched electron transport chain of *E. coli*. When *E. coli* cells are placed in a gradient of reduced versus oxidized quinone they form a distinct band at a specific reduction potential (Bespalov *et al.*, 1996). The higher the reduction potential of the quinone analysed, the stronger the tumbling behavioural response. This means that *E. coli* has a strong repellent response to compounds that divert electrons away from the electron transport chain. This behaviour is termed redox taxis. Remarkably like aerotaxis, this redox taxis response is inverted in *cheB* mutants, and appears to be methylation-independent (Bespalov *et al.*, 1996). Glycerol is also a strong chemoattractant of *E. coli* and response to this electron donor is metabolism-dependent, and methylation-independent (Zhulin *et al.*, 1997). Taxis to glycerol in *E. coli* coincides with an increased membrane potential, indicating that this response is energy-dependent and therefore a true energy taxis.

Although remarkable progress had been made in elucidating both the nature of the aerotactic response in *E. coli* and the stimuli involved in this response, the transducer responsible remained elusive until the *E. coli* genome sequencing project revealed a novel taxis transducer protein that fit the criterion of a true aerotaxis transducer. The gene was initially discovered as an ORF at 69.1 minutes on the *E. coli* genome (Bibikov *et al.*, 1997). The C-terminal end of the predicted translational product contains the HCD region common to transducers while the N-terminal domain contains a sequence of approximately 100 amino acids that bears homology to the oxygen sensing protein NifL of *A. vinelandii* (Bibikov *et al.*, 1997; Hill *et al.*, 1996). This novel domain is now

known to be a PAS domain; these domains are implicated in sensing a variety of stimuli and will be discussed in Section 1.5.3. There is a hydrophobic segment between these two regions that make this transducer a class II transducer. The gene was designated *aer* for *a*erotaxis and *e*nergy *r*esponse. When *aer* is expressed on a multicopy plasmid under the control of its own promoter, cells accumulate in great numbers near air bubbles. Knockout mutants of *aer* do not accumulate near air bubbles but rather appear to avoid oxygen (Bibikov *et al.*, 1997). On glycerol and succinate soft agar plates knockout mutants of *aer* do not move far from the point of inoculation. However, on maltose soft agar plates there is no difference between the movement of *aer* mutants and wild type cells. The transducer Tar governs taxis to maltose, therefore it is not surprising that response to maltose is unaffected in the *aer* mutant. Aer appears to act in a dose dependent manner, as Bibikov *et al.* (1997) noted when *aer* was expressed on a multicopy plasmid taxis to glycerol and succinate was increased. However, when it came to taxis to maltose, the overexpression of *aer* impeded motility compared to wild type cells. This suggests that within a given cell there is an equilibrium of transducer proteins responsible for mediating taxis to a variety of stimuli. When this equilibrium is upset, by the overexpression of one or more transducers or the knockout of a transducer, then cells respond differently, presumably through competition for CheW, CheA and CheY (Bibikov *et al.*, 1997; Rebbapragada *et al.*, 1997)

Further characterization of *aer* revealed that it is a membrane associated protein of approximately 55 kDa. Fluorescence excitation and emission analysis and high performance liquid chromatography analysis also demonstrated that Aer is a flavoprotein that is associated with a FAD cofactor (Bibikov *et al.*, 1997). FAD is bound to Aer non-

covalently and the N-terminal 290 amino acids are required for this binding (Bibikov *et al.*, 2000). FAD binding is essential for the activity of the transducer Aer *in vivo*, as are certain conserved residues in the HCD. Mutants which have the hydrophobic segment replaced with alanine residues do not bind FAD nor do they elicit an aerotactic response. (Bibikov *et al.*, 2000) constructed a hybrid protein of Aer and Tsr (Aesr) containing the N-terminal 290 residues of Aer and the C-terminal sequence of Tsr. This hybrid Aesr transducer was able to effect an aerotactic response suggesting that the N-terminal 290 amino acids of Aer are sufficient for aerosensing and signal transduction.

At the same time that Bibikov *et al.* (1997) were investigating Aer another research group was also studying this novel transducer protein. Rebbapragada *et al.* (1997) used capillary tube assays to investigate aerotaxis in a variety of mutants. In this assay diffusion of oxygen into a liquid filled capillary creates an oxygen gradient. Aerotaxis will lead the cells to a certain oxygen concentration within the gradient, and the bands of cells can be visualised under low magnification. As expected, wild type *E. coli* formed a distinct band in the capillary tubes. The *aer* knockout mutant also formed a band in the capillary tubes, though the band was less defined than the wild type band and formed at a lower oxygen concentration. A *tsr* mutant also formed a band in the tubes, but it was very close to the air bubble, demonstrating that these cells had a greater affinity for oxygen. The most astonishing result was the observation that only in the *aer tsr* double mutant was the aerotactic response completely lost. Similarly, taxis to glycerol was not detected in the *aer tsr* double mutant, though it was observed in the respective single mutants. The implicated role of Tsr in aerotaxis is intriguing, although no mechanism for its ability to sense oxygen, the electron transport chain, or proton motive

force has yet been experimentally established. The only clue to its function is in the work of Dang *et al.* (1986) and their observation that the inverted aerotactic response of *cheB* mutants is not seen in *tsr* mutants. CheB~P has an amidase function, and when it is knocked out Tsr is primarily in an amidated form, it has been proposed that the amidated form of Tsr can sense changes in the proton motive force. Clearly there are mechanisms behind aerotaxis that are not well understood. Further investigation into the nature of Tsr as a transducer for aerotaxis is necessary.

The methylation domains in Aer are not conserved and are not believed to be substrates for either CheB or CheR (Bibikov *et al.*, 1997; Rebbapragada *et al.*, 1997). Taylor *et al.* (1999) suggests that adaptation is not required for cells to form a band in a spatial oxygen gradient. More recently, Bibikov *et al.* (2004) have shown that Aer is not detectibly modified by CheB or CheR, and that Aer was able to mediate aerotaxis in strains that had no other transducers or CheB and CheR, suggesting Aer is a transducer that is methylation-independent.

Homologues of the *E. coli* Aer protein have been identified and characterized in several species including *Pseudomonas putida* (Nichols & Harwood, 2000), *P. aeruginosa* (Hong *et al.*, 2004) and *C. jejuni* (Hendrixson *et al.*, 2001). The *C. jejuni* transducer is unique in that it is composed of two separate proteins, CetA and CetB, which together function as a single aerotaxis transducer.

1.5.3 PAS domains, their role in sensing oxygen and energy

PAS domains have been found in all three domains of life: eubacteria, archaea and eukarya. The name PAS is an acronym of three proteins (*P*er, *A*rnt and *S*im) in *Drosophila* in which imperfect repeat sequences were identified. The list of proteins which these domain are found is extensive and growing but they all have two things in common: they are sensing domains and they are all found intracellularly (Taylor & Zhulin, 1999). They have been implicated in monitoring changes in light, redox potential, oxygen, small ligands, and overall energy level of a cell. In eubacteria and archaea PAS domains are found abundantly in proteins of two-component signal transduction systems. Further characterization of these proteins revealed a conserved sequence of approximately 100-120 amino acids. The knowledge of both the structure and the distribution of PAS domains is undeniably due to the advances in bioinformatics. Most of the work on PAS domains has been done *in silico,* with only a handful of proteins bearing PAS domains having been studied extensively. These include NifL of *A. vinelandii,* Aer of *E. coli*, and photoactive yellow protein (PYP) of *Ectothiorhodospira halophilia.*

The function of various PAS domains is governed by associated co-factors. For example, PYP is a receptor of blue light, carrying a 4-hydrocinnamyl chromophore in its PAS domain (Kyndt *et al.*, 2004); FixL of *S. meliloti* senses oxygen via a heme co-factor (Tomita *et al.*, 2002); and NifL and Aer are both associated with flavin cofactors (Hill *et al.*, 1996; Bibikov *et al.*, 2000). The cofactors are bound by the PAS domain in a structure known as a PAS fold. A predicted consensus structure the PAS fold consists of four ultrastructures: (i) an N-terminal cap, connected to (ii) PAS core, (iii) an α-helical connector and (iv) a β-scaffold. The PAS fold structure resembles a left-handed glove

and the fingers of the glove enclose the cofactor in a hydrophobic core (Taylor & Zhulin, 1999). Mutational studies on Aer demonstrate that the residues in the hydrophobic core of the protein are critical for FAD binding, moreover these appear to be conserved residues in PAS domains in general (Repik *et al.*, 2000).

The big question is how Aer senses changes in the electron transport chain. The most promising explanation has to do with the reducing potential of Aer-bound FAD. The predicted reducing potential of this FAD is in the range that would permit electron exchange with ubiquinone or menaquinone via the electron transport chain (Taylor *et al.*, 2001). When the cell is in need of a terminal electron acceptor, such as oxygen or nitrate, the electron transport chain is fully reduced and therefore one would expect the Aer-bound cofactor to be fully reduced, *i.e.* in the $FADH_2$ state. Conversely if the cell is starved for reducing equivalents such as glycerol or succinate the electron transport chain would be fully oxidized and the flavin co-factor of Aer would be FAD. Both of these situations would be energy limiting for the cell and therefore they would signal a tumbling response. However, when the electron transport chain is functioning at a maximal rate (*i.e.* in an environment with saturating reducing equivalents and optimal electron acceptor concentration), the predominant form of quinone is semiquinone. Therefore the Aer-bound cofactor would be FADH, and this would signal for a smooth swimming response (Taylor *et al.*, 2001). There is no experimental evidence to support this model, however, there appears to be a positive correlation between the number of PAS domains per genome and the number of predicted proteins involved in electron transport (Zhulin & Taylor, 1998).

1.6 Complex pattern formation by motile bacteria

One of the most spectacular phenomena in relation to bacterial motility are the complex patterns formed by bacteria under specific conditions. Elena Budrene discovered that when motile *E. coli* are inoculated into liquid or soft agar containing certain intermediates of the tricarboxylic acid cycle (succinate, malate and fumarate) they aggregate in highly structured patterns (Budrene & Berg, 1991). These patterns are remarkable both in their symmetry and in the apparent multicellular behaviour exhibited by the cells. It was postulated that the cells were aggregating in response to an attractant that they excrete. The excretion of this attractant could be triggered by subjecting the cells to oxidative stress (Budrene & Berg, 1991). It was subsequently determined that the excreted attractant is aspartate and the transducer Tar, the sensor for aspartate, is required for the aggregation behaviour (Budrene & Berg, 1995).

The *E. coli* cells create a gradient of attractant through uptake and metabolism of compounds in the media. Cells move up this gradient and can be observed as a large ring expanding radially. If the cells excrete an attractant, like aspartate, then the ring breaks up into discrete aggregates (Berg, 1996). The initial concentration of substrate determines both the cell density and the final concentration of excreted attractant (Budrene & Berg, 1995). Fluorescence microscopy examining the behaviour of *E. coli* cells within the aggregates has shown that the cells' tumble frequency is dependent upon its position within the aggregate. At the center of the aggregate the tumbling behaviour is suppressed so the cells swim until they reach the edge of the aggregate. At the aggregate

edge the cells will tumble, thereby maintaining the sharp boundary of the aggregate and preventing the cell from leaving the cluster (Mittal *et al.*, 2003).

The experimental conditions required to generate the patterns are stringent, very different patterns were reported for 1 mM and 3 mM succinate plates, and different strains responded differently (Budrene & Berg, 1995). It was reported that strains deficient in the transducer Tsr yielded the most reproducible patterns (Budrene & Berg, 1991). The thickness of the semi-solid agar layers also had to be between 2-3 mm in order to yield patterns (Budrene & Berg, 1991).

Despite the strict conditions required to generate patterns this phenomenon has been described in *S. typhimurium* (Blat & Eisenbach, 1995; Budrene & Berg, 1995) and a *Pseudomonas* strain (Emerson, 1999). As with *E. coli,* the patterns produced by *S. typhimurium* can be generated by growing the cells on media containing succinate, fumarate or malate (Budrene & Berg, 1995). Blat and Eisenbach (1995) demonstrated that *S. typhimurium* will autoaggregate when inoculated into tryptone-based complex media. These patterns are less stable than the tricarboxylic acid-generated patterns and do not require the Tar transducer. *Pseudomonas* strain KC was also shown to aggregate, but the substrates required for aggregation were nitrate and nitrite (Emerson, 1999).

Several mathematical models have been derived which attempt to explain the patterns in *E. coli* (Brenner *et al.*, 1998; Tyson *et al.*, 1999; Tyson *et al.*, 1999). All the models are based on the concept of cells aggregating in response to an attractant that they excrete. For the most part these models work very well, however there is no model for the *Pseudomonas* patterns described by Emerson (1999) or the Tar-independent patterns described by Blat and Eisenbach (1995).

1.7 Thesis objectives

There are two main objectives of this thesis: to investigate the taxis transducer proteins in *A. vinelandii* and to study the phenomenon of complex pattern formation by this organism. Bioinformatics was used to initially identify and characterize, *in silico,* the transducers. All the transducers in the *A. vinelandii* genome, including two PAS-bearing transducers, were knocked-out by gene disruption. A soft agar swimming assay, adapted for *A. vinelandii*, was developed to study the motility of wild-type and mutant cells. The soft agar assay was used because it is a flexible assay that yielded reproducible quantifiable results. This method also led to the discovery of complex pattern formation by *A. vinelandii*. This behaviour was further investigated and the effect of the transducer mutations on the pattern formation was also studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 2.1. *Azotobacter vinelandii* strains were cultured at 28°C in Burks buffer (BB, 0.81 mM $MgSO_4$, 0.58 mM $CaSO_4$, 5 μM Na_2MoO_4 and 12.5 μM $FeSO_4 \cdot 7H_2O$) containing 1% glucose (w/v) and 1.1 g ammonium acetate per liter (BBGN). The *A. vinelandii* cultures were supplemented with kanamycin (10 μg/ml), streptomycin (1 μg/ml) and tetracycline (3 μg/ml) as required. *A. vinelandii* strains were routinely grown on agar slants that contained 5 ml of BBGN solidified with 1.5% (w/v) agar.

E. coli strains were cultured at 37°C in Luria-Bertania (LB) media (10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 10 g/l NaCl) supplemented with ampicillin (50 μg/ml), kanamycin (30 μg/ml), spectinomycin (100 μg/ml), and tetracycline (10 μg/ml) as required.

2.2 Bioinformatic analysis

For *in silico* analysis of the DNA sequences of interest GeneTools 2.0 (BioTools, University of Alberta) was used. This program was used to identify restriction enzyme sites, design primers, identify open reading frames and analyse sequencing results. *A. vinelandii* genomic DNA sequences were obtained from http://ava.biosci.arizona.edu/ (accessed many times from June 2002-September 2004). The program SMART (Simple Modular Architecture Research Tool), available through http://smart.embl-heidelberg.de,

Table 2.1 List of plasmids and bacterial strains used in this study

Strains and Plasmids	Description	Reference or source
A. vinelandii strains		
UW (also known as OP)	*aerB,PH+, algU-, tlpA,B,C,D,E,F+*	
Δ*aerPH*	Δ*aerPH*::ΩSm^R, *aerB+, algU-*	This work
aerP	*aerP*::Km^R, *aerH,B+, algU-*	This work
aerH	*aerH*::ΩSm^R, *aerP,B+, algU-*	This work
aerB	*aerB*::Ω,Tc^R *aerPH+, algU-*	This work
aerB,PH	*aerB*::ΩTc^R, Δ*aerPH*::ΩSm^R, *algU-*	This work
tlpA	*tlpA*::ΩSm^R	This work
tlpB	*tlpB*::ΩSm^R	This work
tlpC	*tlpC*::ΩSm^R	This work
tlpD	*tlpD*::ΩSm^R	This work
tlpE	*tlpE*::ΩSm^R	This work
tlpF	*tlpF*::ΩSm^R	This work
E coli strains		
DH5α	General cloning strain	

Plasmids		
pHP45Ω	Source of ΩSm^R Spe^R cassette (hereafter referred to as ΩSm^R)	Prentki & Krisch, 1984
pHP45Ω-Tc	Source of ΩTc^R cassette	Fellay *et al.*, 1987
p34S-Km3	Source of Km cassette	Dennis & Zylstra, 1998
pCR®2.1-TOPO	PCR product cloning vector	Invitrogen Life Technologies
pDrive	PCR product cloning vector	Qiagen
pGEM®-T Easy	PCR product cloning vector	Promega
pHW1	3,254-bp PCR fragment containing *aerP aerH* and *fdxA* in pCR®2.1-TOPO	This work
pHW2	ΩSm^R cassette inserted between two *Sma*1 sites on pHW1, removing a 1,368-bp fragment of aerPH	This work
pHW3	ΩSm^R cassette inserted into a unique *Nde*1site on pHW1, disrupting *aerH*	This work
pHW4	1,026-bp PCR fragment of *aerP*, with *Eco*R1 and *Hind*III restriction sites designed in the primer sequence, in pDrive	This work
pHW5	1,020-bp *Eco*R1-*Hind*III product of pHW4 cloned in–frame into pRSET-B.	This work

pHW6	2,119-bp PCR fragment containing *aerB* in pDrive	This work
pHW7	ΩTc^R cassette inserted into unique *Nco*1 site within *aerB* in pHW6 (non-polar orientation)	This work
pHW8	1,446-bp *Eco*R1-*Pst*1 fragment of pHW1 carrying the upstream sequence and 5'-end of *aerP* cloned into the corresponding restriction sites in pDK6	This work
pHW14	1,879-bp PCR fragment of *tlpA* in pGEM®-T Easy	This work
pHW15	1,930-bp PCR fragment of *tlpB* in pGEM®-T Easy	This work
pHW16	1,863-bp PCR fragment of *tlpC* in pGEM®-T Easy	This work
pHW17	1,732-bp PCR fragment of *tlpD* in pGEM®-T Easy	This work
pHW18	1,759-bp PCR fragment of *tlpE* in pGEM®-T Easy	This work
pHW19	1,788-bp PCR fragment of *tlpF* in pGEM®-T Easy	This work

pHW20	ΩSm^R cassette inserted into a unique *Eco*RV site on pHW14, disrupting *tlpA* (non-polar orientation)	This work
pHW21	ΩSm^R cassette inserted into a unique *Eco*47III site on pHW15, disrupting *tlpB* (non-polar orientation)	This work
pHW22	ΩSm^R cassette inserted into a unique *Eco*RV site on pHW16, disrupting *tlpC* (non-polar orientation)	This work
pHW23	ΩSm^R cassette inserted into a unique *Eco*RV site on pHW17, disrupting *tlpD* (non-polar orientation)	This work
pHW24	ΩSm^R cassette inserted into a unique *Eco*47III site on pHW18, disrupting *tlpE* (non-polar orientation)	This work
pHW25	ΩSm^R cassette inserted into a unique *Eco*47III site on pHW19, disrupting *tlpF* (non-polar orientation)	This work

was used to identify conserved protein domains and generate diagrams showing domain composition of proteins of interest. The program ClustalW, available through http://www.ebi.ac.uk/clustalw/, was used to build multiple protein sequence alignments and compute neighbor-joining phylogenetic trees.

2.3 Molecular biology techniques employed for construction of plasmids and mutant strains

2.3.1 PCR and sequencing

All PCR reactions were set up using Taq DNA polymerase, provided by Dr Pickard (University of Alberta). A standard 50 µl reaction contained the following: 4 µl 5 M Betaine, 5 µl 10X Stemke buffer (SB, 0.7 M Tris, pH 8.8, 40 mM $MgCl_2$, 1% Triton-X-100, 1 mg/ml BSA), 5 µl 1 mM dNTPs (Roche), 0.75 pmol forward primer, 0.75 pmol reverse primer, template DNA (concentration varied up to 1 µg), 5 µl Taq DNA polymerase diluted in 1X SB (added after initial denaturation), and sterile Milli-Q dH_20 to adjust the volume to 50µl. Table 2.2 lists all the primers used in this study.

A Techne Techgene thermocycler was used for most PCR and sequencing reactions. Standard PCR reaction conditions began with a 5-minute initial denaturation at 95°C, followed by a 68°C hot start at which time the Taq DNA polymerase was added. The reactions then ran through 25-35 cycles as follows: denaturation at 95°C for 30-seconds, annealing temperature (varied) for 30°C, elongation at 68°C for 1 minute for

Table 2.2 List of DNA oligonucleotides used in this study

Primer name* (WJP#)	Sequence (5' 3')	Description	Used to make plasmid
74	ggtgcgtcgtttcggtctgc	*rpoS* forward	pHW1
169	ccccagccgccgatccagagc	*mutS* reverse	pHW1
206	cgggaagcttgctcggtgcgcctggacag	Upstream *aerP* with *Hind*III forward	pHW4
207	gcgcgaattccactccctgcatgcctatctc	Downstream *aerP* with *Eco*R1 reverse	pHW4
233	gccgggccagacgctccaggtact	Upstream *aerB* reverse	pHW6
225	cgggcggcggaggacggaggtatc	Downstream *aerB* forward	pHW6
250	aggggcaagggctccaaggatcg	Sequencing primer reading out of Ω Sm^R cassette	
297	cggcaaggggagcgcggattca	*tlpA* forward primer	pHW14
298	cgccctgtcctgttggtggtgatgtt	*tlpA* reverse primer	pHW14
299	cggcgaacatcatcaccaacaggac	*tlpB* forward primer	pHW15
300	gcccgcgatacctccgtcctc	*tlpB* reverse primer	pHW15
301	ggcggtcgctgcgggaaaagg	*tlpC* forward primer	pHW16
302	cgccgaacgccgctgctccg	*tlpC* reverse primer	pHW16
303	gcgggatttttttgcgtggcttgag	*tlpD* forward primer	pHW17
304	ccccggtcaaaactcattccactg	*tlpD* reverse primer	pHW17
305	gctcgacgcggcgctctactac	*tlpE* forward primer	pHW18
306	gccgctgccgctcgaccatgc	*tlpE* reverse primer	pHW18
307	acgcgtggctttcctggtgacc	*tlpF* forward primer	pHW19
308	gggcgaggaggttggtctggaa	*tlpF* reverse primer	pHW19

*All primers except WJP74 and WJP169 designed by HLW

every Kbp amplified. The final step in the PCR reaction was a final elongation at 68°C for 10 minutes. PCR reactions were promptly removed from the thermocycler and stored at 4°C

Sequencing was completed using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Sequencing reactions were set up according to manufacture's protocols as follows: 200-700 ng template plasmid, 5 pmol primer, 8 µl ET mix and sterile Milli-Q dH_20 to adjust the volume to 20 µl. Due to the high GC content of *A. vinelandii* DNA a two-step sequencing reaction combining the elongation and annealing steps was typically used. A standard sequencing reaction of 30-35 cycles was as follows: denaturation at 95°C for 20-seconds followed by elongation at 60°C for 1-minute. When cycling was complete 2 µl 1.5 M sodium acetate containing 250 mM EDTA buffer was added to the reaction followed by 80 µl of 95% ethanol. Samples were then centrifuged at maximum speed in a microcentrifuge at room temperature or 4°C for 15 minutes. The supernatant was carefully removed with a pipette and the pellets were washed with 200 µl of 70% ethanol. The pellets were allowed to dry and the samples were delivered to the Molecular Biology Service Unit (University of Alberta, Department of Biological Sciences) for resuspension, electrophoresis and analysis of the DYEnamic ET terminators.

2.3.2 Agarose gel electrophoresis of DNA

DNA fragments were analysed by agarose gel electrophoresis using gels consisting of 0.7-1.5% agarose. TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0)

was used both in the preparation of the gels and as the electrophoresis buffer. Prior to loading the sample on the gel 5X DNA loading buffer (0.125% bromophenol blue, 30% sucrose, 5 mM EDTA pH 8.0) was added to the sample to a final concentration of 1X. Commercially prepared 1Kbp DNA ladder (New England Biolabs) was loaded onto the gels to assist in size determination of linear DNA fragments. A constant voltage was applied to the gels, ranging from 15 to 200 V, over a varying period of time. Upon completion of electrophoresis gels were stained in ethidium bromide (10 µg/ml in TAE) for 15-20 minutes and DNA was visualized on a UV transilluminator.

2.3.3 DNA isolation and purification

Plasmid DNA was isolated from *E. coli* using the alkaline-lysis method described by (Sambrock & Russel, 2001) In general 5 ml of overnight *E. coli* culture was processed and the final DNA pellet was resuspended in 20 µl DNA storage buffer (10 mM Tris, 1 mM Na_2EDTA, 40 µg/ml RNase A, pH 8.0).

Genomic DNA was isolated from *A. vinelandii* using a modified CTAB-sodium chloride method as described by Ausubel *et al.* (1995). The modification of this method involved recovering the bacterial cells from an agar slant instead of harvesting an overnight culture. The final genomic DNA pellet was resuspended in 100 µl DNA storage buffer.

DNA fragments were purified from agarose gels using one of two commercially available kits, the GENECLEAN kit (Bio 101) or the Agarose Gel DNA Extraction Kit

(Roche). The manufacturer's protocols were followed and the isolated DNA fragments were resuspended in 10-50 µl of DNA storage buffer or water.

For short-term storage DNA solutions were left at room temperature or at 4°C, for prolonged storage DNA was kept at -20°C.

2.3.4 Enzymatic manipulation of DNA

Plasmid DNA was routinely digested with restriction endonucleases for plasmid DNA restriction analysis or cloning purposes. The restriction endonucleases used were purchased from Roche or New England Biolabs. The amount of DNA digested as well as the total volume of the restriction digests varied. The reactions were set up using the buffer supplied by the manufacturer and containing an amount of restriction enzyme that did not exceed 0.1 volume of the total reaction mixture. Reactions were regularly incubated for 1 hour at the proper incubation temperature. If the restriction digest was required for an immediate downstream ligation then the restriction enzyme was inactivated with heat treatment or removed by extraction with one volume phenol: chloroform: isoamyl alcohol (25:24:1).

Klenow DNA polymerase was used to convert 3' recessed sticky ends (generated by digestion with *Nde*I and *Nco*I) to blunt ends. The reactions were set up as follows: digested DNA (concentration varied), 1 mM each of desired dNTPs (as sequence required), 2 µl 10X filling buffer (500 mM Tris pH 7.5, 100 mM $MgCl_2$, 10 mM DTT, 500 µg/ml BSA) 1 µl (1 U) Klenow (Roche), Milli-Q H_2O to a final volume of 10 µl.

The reactions were incubated at 37°C for 15 minutes, followed by heat inactivation at 65°C for 10 minutes.

Prior to ligation of DNA fragments all DNA samples were incubated at 42°C for 10 minutes to ensure DNA in solution was thoroughly solubilized. Reactions were set up to have a final volume of 10 µl containing varying molar ratios of vector: insert DNA, 1 µl 10X ligation buffer (660 mM Tris pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP) followed by 1 µl of T4 DNA ligase (Roche). Blunt and sticky end ligations were left at room temperature overnight. For some ligations the Rapid DNA Ligation Kit (Roche) was used according to manufacturer's protocols.

2.3.5 Cloning of PCR products

PCR products of interest that had been gel purified, as described above, were cloned into commercial PCR cloning vectors. The vectors used were pCR®2.1-TOPO (Invitrogen Life Technologies), pDrive (Qiagen), and pGEM®T Easy (Promega) (see Table 1). Ligation reactions were set up as per the manufacturers protocols and the ligations were left at room temperature for 3 hours or 4°C overnight. The ligations reactions were then transformed into *E. coli* as described below.

2.3.6 Transformation of bacteria

i *E. coli* transformations

E. coli DH5α cells were prepared for transformation using the 18°C method as described by (Sambrock & Russel, 2001). Competent cells were flash-frozen in liquid nitrogen and stored at –80°C for up to 3 months. Transformations were carried out by adding 50 µl of competent cells, thawed on ice for up to 30 minutes, to 1.5 ml Eppendorf tubes containing the DNA solution to be transformed. The concentration of DNA varied but the volume never exceeded 5 µl. The cell-DNA mixture was incubated on ice for 30-minutes; followed by a heat shock at 42°C for 90 seconds. After 2 minutes on ice 1 ml LB medium was added to the tubes which were then incubated at 37°C for 1 hour to allow the expression of antibiotic resistance genes. A portion of the cell suspension (100-200 µl) was then plated out onto antibiotic selective medium. The remainder of the cell suspension was pelleted in a microcentrifuge for 1 minute, resuspended in a smaller volume of supernatant, and plated out on antibiotic selective media. In some cases the transformants were screened for β-galactosidase activity (blue-white differentiation); in these cases the cell suspensions were plated onto antibiotic selective medium containing 0.2 mM IPTG and 40 µg/ml X-gal. Transformation plates were incubated overnight at 37°C and examined for isolated transformant colonies.

ii *A. vinelandii* transformations

A. vinelandii UW were induced to natural competence under iron-limited conditions, as described by (Page & von Tigerstrom, 1978 and 1979). The *A. vinelandii* UW strain of interest was inoculated onto the surface of a BBGN slant and incubated at 30°C until the slant turned green (1-3 days), an indication of iron-limitation. The cells

were gently rinsed from the surface of the slant with 1 ml MOPS buffer (16 mM $MgCl_2$ 20 mM MOPS, pH 7.2). Plasmid or genomic DNA for transformation was resuspended in MOPS buffer and this solution was mixed with the iron-limited cell suspension in a 1:1 ratio (250-500 µl). Several aliquots (100 µl) of the DNA-cell mixture were spotted onto a BBGN plate and left agar-side down at 30°C overnight. The following day a sterile 200 µl pipette tip was used to scrape up one of the spots and the cell mass was resuspended in 100 µl of MOPS buffer. The cell suspension was subsequently plated out on antibiotic selective BBGN agar plates and left to incubate at 28°C. The spots were scraped up and harvested in this way for up to 7 days. The antibiotic selective BBGN plates were examined for isolated transformant colonies for up to 4 days. A negative control containing no DNA was always included to ensure the efficacy of the antibiotic selection and the purity of the solutions. Once transformants were isolated they were maintained and stored on antibiotic selective media.

2.3 Soft agar swimming assays

Soft agar plates were made with modified BB, with 0.58 mM $CaCl_2$ substituted for the $CaSO_4$ normally present. This modified medium contained fewer insoluble particles and was more transparent than regular BB. Low ash noble agar (Difco) at concentrations of 0.2-0.5% was used to gel the plates. Whenever possible carbon sources were made up as 1 M stocks in BB and when necessary the pH was adjusted to 7.0 with NaOH. Carbon sources were aseptically added to the medium just prior to plate pouring. Amino acids and other nitrogen sources were made as filter sterilized 100 mM stocks in

BB. Amino acids were added prior to or after autoclaving, depending upon experimental conditions. In some experiments where the amount of carbon or nitrogen source varied, the amount of BB also varied so that all of the plates had the same volume. Tetrazolium red (2,3,5-triphenyl-tertrazolium chloride) was sometimes added as a vital dye to increase contrast and assist in capturing the images, a sterile 1% solution was a 1000X concentrated stock and was added just prior to plate pouring.

Once the medium was prepared, 25 ml was poured into sterile 10 cm diameter Petri-plates. Plates were left undisturbed to gel for 1-2 hours at room temperature. For the soaked disc assays, sterile discs (Schleiche & Schuell Inc. 740-E 12.7 mm diameter) were soaked in the compound to be tested and aseptically transferred to the soft agar surface. A pipette was used to inoculate the plates with 2-10 µl of a dense cell suspension, usually recovered from an agar slant. Cells were recovered in 1-1.5 ml Chemotaxis Buffer (potassium phosphate 10 mM pH 7.0 potassium EDTA 1 mM). Plates were incubated at 28°C and images are captured with or the Syngene bioimaging system.

2.4 Aerotaxis assays

Aerotaxis was investigated using optically flat microslides (inner dimensions 0.1 by 2 by 50 mm, VitroCom Inc.) A light suspension of motile bacteria in BBGN medium was introduced into the microslides by capillary action and incubated at 28°C for 10-15 minutes. The microslides were examined for bacterial band formation at or near the air liquid interface using dark field microscopy.

CHAPTER 3

RESULTS

3.1 Identification and bioinformatics analysis of taxis and motility related sequences in the *A. vinelandii* UW

The initial identification of an energy taxis transducer gene in the *A. vinelandii* UW genome was an unexpected result that developed from the search for the stationary phase sigma factor gene *rpoS*. A 2,743 bp *Xho*1 fragment bearing the 3' end of the *pcm* gene (L-isoaspartate o-methyltransferase) the complete *nlpD* gene (murein endopeptidase) and the 5' end of the *rpoS* gene was isolated and sequenced (A. Tindale and S. Kujat-Choy.). In order to obtain the complete sequence of the *rpoS* gene PCR primers were designed to amplify the region between the 5' *rpoS* gene and *mutS* gene. The *mutS* gene was selected for this purpose because it was known to be located downstream of the *rpoS* gene in other bacteria (Herbelin *et al.*, 2000; Kotewicz *et al.*, 2003) and the sequence of the *A. vinelandii* UW *mutS* gene was available (Le *et al.*, 1993). Early PCR and sequence analysis of the region between *rpoS* and *mutS* revealed two separate ORFs encoding a transducer system believed to be involved in energy taxis (A. Tindale, S. Kujat-Choy, and D. Meakins). The upstream gene encodes a 315-residue product that contains a PAS domain. This gene was designated *aerP* (aerotaxis and energy response with PAS domain). The second gene, encodes a 263-residue product with a high identity to the highly conserved domain (HCD) of transducer proteins. This gene was designated *aerH* (aerotaxis and energy response with HCD domain). The region was sequenced again and the two genes *aerP* and *aerH* were confirmed to be interrupted by a stop codon (H. Whelan). The entire 5,876 bp region from *pcm* to *mutS* was submitted to Genebank (accession number AF421351, May 2002). This sequence

was subsequently confirmed when the initial genomic data for *A. vinelandii* UW was released by the Joint Genome Institute, JGI (June 2002).

It was postulated that *aerP* and *aerH* may function together as a bipartite energy taxis system, as identified in *C. jejuni* (Hendrixson *et al.*, 2001). For this reason throughout this work the two genes will be referred to as *aerP* and *aerH* and the resulting proteins will be referred to jointly as AerPH.

Once the initial genome sequence of *A. vinelandii* UW became available, seven other transducer genes were identified. Identification of the transducer genes was accomplished by BLAST search analysis of the *aerH* HCD against the draft genomic sequence. Because of the remarkable homology of this domain, the other transducers were easily identified with e-values ranging from e-110 to e-70. Among the transducers identified was a second PAS-domain-bearing energy taxis transducer gene that encodes a 569-residue product; this gene was designated *aerB*. Unlike the *aerP* and *aerH* genes, *aerB* contains the PAS and HCD domains in the same ORF. The six remaining transducer sequences do not encode PAS domains and appear to encode more classical transducer sequences. These classical transducers were designated *tlpA, tlpB, tlpC, tlpD, tlpE* and *tlpF* (for transducer-like protein).

The arrangement most of the transducer genes within the *A. vinelandii* UW genome is shown in Figure 3.1. The *aerP* and *aerH* genes are located between the variable *rpoS-mutS* locus of the chromosome (Figure 3.1A). The *aerPH* genes are located in opposite orientation to the gene encoding the iron-sulphur protein ferredoxin A (*fdxA*). No other transducers proteins are encoded nearby. This region is known to contain variable sequences among *Azotobacter* species (Page lab, unpublished results)

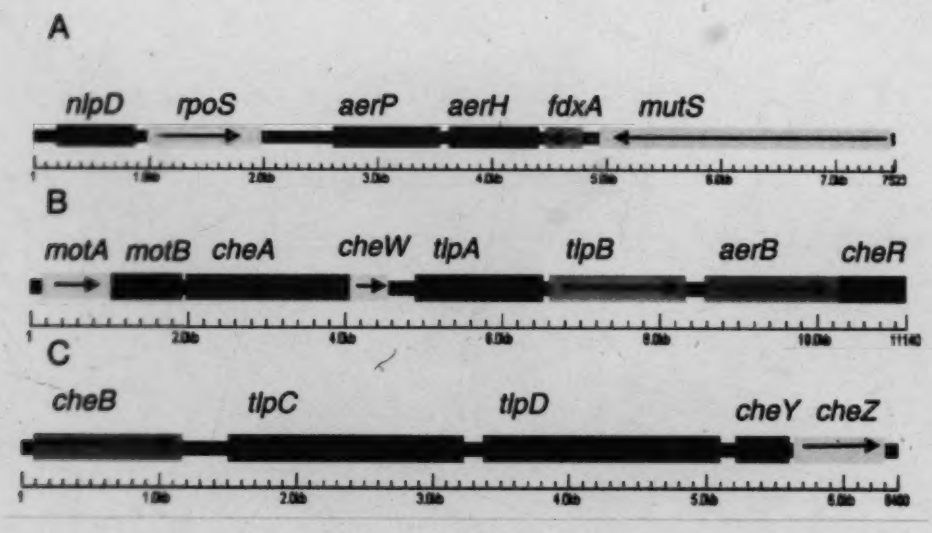

Figure 3.1 Chemotactic operons identified in the genome of *A. vinelandii* UW. (A) A bipartite energy taxis system, *aerP* and *aerH*, located between *mutS* and *rpoS*. (B) Orthologue to the Mocha operon of *Salmonella* and E.*coli*. (C) Meche operon

and in several other bacterial species (Ferenci, 2003). It appears that in the closely related *A. salinestris* the two genes, *aerP* and *aerH*, are not interrupted by a stop codon and are present in a single ORF, designated *aerS* (J. Robitaille).

Two separate chemotactic operons were also identified and are shown in Figure 3.2B and C. The genes *tlpA*, *tlpB* and *aerB* are located together downstream from *cheW* and upstream from *cheR*. This operon also contains the *motA*, *motB*, and *cheA* genes. The second major chemotaxis operon encodes *cheB*, *tlpC*, *tlpD*, *cheY* and *cheZ*. These two operons are similar to Mocha and Meche chemotactic operons, respectively, of *Salmonella* and *E. coli* (Stock & Surette, 1996). The two remaining transducers, *tlpE* and *tlpF*, are encoded separately from one another elsewhere in the chromosome. There were no duplicate copies of any of the *che* genes, as has been observed in other organisms.

The SMART program was used to identify and illustrate conserved domains and transmembrane segments in the transducers proteins (Figure 3.2). The energy taxis proteins AerB and AerP both contain the PAS domain and the *P*AS *a*ssociated *C*-terminal domain (PAC). The HAMP domain, a domain common to many bacterial signal transduction proteins, was also found in all of the transducers with the exception of the AerPH proteins. Of all the classical transducers TlpE is the largest and contains a very large N-terminal cytoplasmic domain, the function of which is unknown.

A multiple alignment of the amino acid sequence of AerB, AerPH and 44 other PAS-bearing energy taxis genes from different bacteria was generated using ClustalW. Table 3.1 lists all the transducers used to construct the multiple alignment along with the number of PAS, PAC, HAMP, HCD and transmembrane domains identified in each transducer protein. The transducers were identified on various

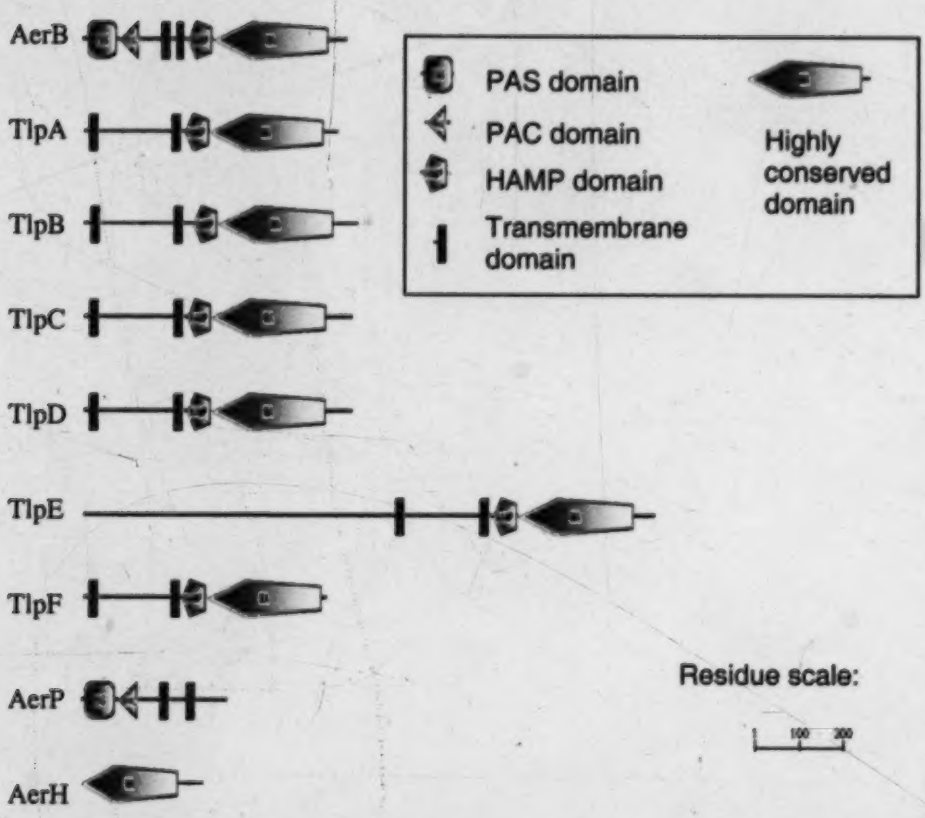

Figure 3.2 Domain composition and organization of all taxis transducer proteins in the *A.vinelandii* UW genome. Diagrams were generated using the SMART program (Simple Modular Architecture Research Tool)

Table 3.1 PAS-bearing taxis transducer proteins identified on sequence databases. The accession numbers to the proteins are shown as are the number of PAS, PAC, HAMP HCD and TM domains. TM domains identified using TMHMM 2.o program or SOUSI program, all other domains identified based on SMART analysis.

Organism	Accession number	PAS	PAC	HAMP	HCD	TM
Azotobacter vinelandii (AerP)	Q8L317	1	1	0	0	2
Azotobacter vinelandii (AerH)	Q8L316	0	0	0	1	0
Azotobacter vinelandii (AerB)	NA	1	1	1	1	2
Azotobacter salinestris (AerS)	NA	1	1	1	1	2
Agrobacterium tumefaciens (pTi-SAKURA ORF114)	Q9R6E5	2	2	1	1	0
Agrobacterium tumefaciens (pTi-Washington C58 McpA)	Q8U611	2	2	1	1	0
Agrobacterium tumefaciens (Washington C58 atu3094)	Q8UBC2	2	2	1	1	0
Agrobacterium tumefaciens (Washington C58 atu2618)	Q8UC80	2	2	1	1	0
Archaeoglobus fulgidus (TLPC-1)	O29228	2	2	1	1	0
Archaeoglobus fulgidus (TLPC-2)	O29217	3	3	1	1	0
Bradyrhizobium japonicum	Q89R39	1	1	1	1	2
Campylobacter jejuni (CetB)	Q9PNA6	1	0	0	0	0
Campylobacter jejuni (CetA)	Q9PNA5	0	0	1	1	1?
Caulobacter crescentus (McpH)	Q9A357	2	2	1	1	0
Chromobacterium violaceum (Aer)	Q7P118	1	1	0	0	0
Chromobacterium violaceum (McpIV)	Q7P119	0	0	0	1	2
Desulfovibrio vulgaris (DcrA)	P35841	1	1	1	1	2

Escherichia coli (K12 Aer)	P50466	1	1	1	1	2
Escherichia coli (O157:H7 Aer)	Q8X4Z3	1	1	1	1	2
Escherichia coli (O6 Aer)	Q8FDF8	1	1	1	1	2
Escherichia coli (JM109 Tn1721 ORF1)	Q56321	1	1	1	1	2
Leptospira interrogans (Aer)	Q8F9Y8	1	1	0	1	2
Methanosarcina acetivorans (Mcp1)	Q8TLG5	2	1	1	1	0
Methanosarcina mazei (TlpC)	Q8PWD1	2	2	1	1	0
Methanosarcina mazei (Mcp)	Q8Q005	2	2	1	1	0
Nitrosomonas europaea	Q82TM2	2	1	1	1	2
Pseudomonas aeruginosa (PAO1 Aer)	Q9I3F6	1	1	1	1	2
Pseudomonas putida (PRS2000 Aer)	Q9WWT4	1	1	0	1	2
Pseudomonas putida (KT2440 Aer-1)	Q88KN3	1	1	0	1	0
Pseudomonas putida (KT2440 Aer-2)	Q88L25	1	1	0	1	2
Pseudomonas putida (KT2440 Aer-3)	Q88EE4	1	1	0	1	1?
Pseudomonas resinovorans (pCar1 CA10 ORF183)	Q8GHL8	1	1	0	0	0
Pseudomonas resinovorans (pCar1 CA10 ORF182)	Q8GHL9	0	0	1	1	0
Pseudomonas stutzeri	Q84IR1	0	1	0	1	0
Pseudomonas syringae (DC3000 Aer-1)	Q886D1	1	1	0	1	2
Pseudomonas syringae (DC3000 Aer-2)	Q884S4	1	1	0	1	2
Ralstonia eutropha	NA	1	1	1	1	2
Ralstonia solanacearum (megaplasmid RS03168)	Q8XQK0	1	1	1	1	1?
Ralstonia solanacearum (megaplasmid RS03711)	Q8XT60	1	1	1	1	2

Rhizobium meliloti (1021 McpY)	Q92LN2	1	2	1	1	0
Rhizobium meliloti (RU11/001 McpY)	Q9EZD6	1	2	1	1	0
Salmonella enterica sv. Typhi (Aer)	Q8Z3M0	1	1	0	1	1?
Salmonella typhimurium (LT2 Aer)	Q8ZLX8	1	1	1	1	2
Shewanella oneidensis	Q8EBU5	1	1	1	1	2
Vibrio cholerae (E1 Tor VC0521)	Q9KUK6	1	1	1	1	2
Vibrio cholerae (E1 Tor VC0098)	Q9KVP3	2	2	1	1	0
Yersinia pestis (bv. Mediaevalis)	Q8D0P9	1	1	1	1	1?
Yersinia pestis (bv. Orientalis)	Q8ZFP9	1	1	1	1	1?

? Indicates proteins with only one predicted TM domain. There are likely two domains in these proteins and programs are unable to distinguish them due to their proximity.

databases including Swiss-Prot, TrEMBL, SMART and the bacterial genomes available at JGI, and the available accession numbers are listed in Table 3.1. The multiple alignment identified, as expected, the highest homology within the highly conserved domains. There are 21 columns in the HCD alignment where the residues are completely conserved (Figure 3.3). The PAS domains also aligned with one another, although they did not show the same homology observed in the HCD, although two columns do have relatively conserved hydropathic residues characteristic of this domain (Figure 3.4).

There are numerous transducers identified on the databases that have no predicted transmembrane domains. These putatively cytoplasmic transducers are found in: *R. solanacearum*, *A. tumefaciens*, *R. meliloti*, *C. cresentus*, *V. cholerae*, *A. fulgidus*., *M. acetovorans*, *M. mazei*, *P. putida* and *P. resinovorans*. Many of these cytoplasmic transducers have more than one PAS domain, with as many as 3 PAS domains found in a single transducer *A. fulgidus* TLPC-2 (Table 3.1).

Three other bipartite energy taxis system similar to AerPH of *A. vinelandii* were also used in the multiple alignment. These include transducers from *C. jejuni*, *P. resinovorans*, and *C. violaceum*. For the construction of the multiple alignment all the bipartite systems were treated as single proteins.

ClustalW was also used to construct a phylogenetic tree based on the neighbour-joining method from the multiple alignment (Figure 3.5). In this tree the sequences of AerB and AerPH are very closely related to one another. AerS also clustered with the *A. vinelandii* AerPH proteins. Other closely related taxis transducer proteins to the *Azotobacter* sequences are from the beta subdivision proteobacteria *R. eutropha*, and *N. europaea*. Most of the transducers in the tree tend to cluster with transducers from the

```
A.vinelandii_AerPH/371-424          EIIRVIDSIAFQTNILALNASVEAARAGEHGRGFAVVASEVRNLATRSAA
A.vinelandii_AerB/373-426           EIIDVIDSIAFQTNILALNASVEAARAGEQGRGFAVVAGEVRNLASRSAE
A.salinestris_AerS/395-448          EIIQVIDSIAFQTNILALNASVEAARAGEHGRGFAVVASEVHNLATRSAA
A.tumefaciens_pTiSAKURA/391-44      NIIGVIDEIAFQTNLLALNAGVEAARAGEAGKGFAVVAQEVRELAQRSAK
A.tumefaciens_pTi_C58/396-449       NIIGVIDEIAFQTNHLALNAGVEAARAGEAGKGFAVVAQEVRELAQRSAK
A.tumefaciens_C58_atu3094/391-      NIIGVIDEIAFQTNLLALNAGVEAARAGEAGKGFAVVAQEVRELAQRSAT
A.tumefaciens_C58_atu2618/391-      NIIGVIDDIAFQTNLLALNAGVEAARAGEAGKGFAVVAQEVRELAQRSAV
A.fulgidus_TLPC1/470-523            KVTERIKSIADQTNLLALNAAIEAARAGEHGRGFAVVADEVRKLAEESPK
A.fulgidus_TLPC2/627-680            KVTERIKSIADQTNLLALNAAIEAARAGEHGRGFAVVADEVRKLAEESRK
B.japonicum/398-451                 DVVELINAIAGQTNLLALNATIEAARAGEAGRGFAVVASEVKALAEQTAK
C.jejuni_CetBA/403-456              SVVEVIPDIADQTNLLALNAAIEAARAGEHGRGFAVVADEVRKLAERTQR
C.crescentus_HcpH/423-476           QIISVIDEIAFQTNLLALNAGVEAARAGEAGRGFAVVAQEVRALAQRSAE
C.violaceum_Aer_HcpIV/440-493       TSLADISAIADQTNLLALNAAIEAARAGETGRGFAVVADEVRKLAERSKS
D.vulgaris_DcrA/501-554             RVIEVINEIADQTNLLALNAAIEAARAGDAGRGFAVVADEVRKLAEKTHV
E.coli_K12_Aer/353-406              TITSLINDIAFQTNILALNAAVEAARAGEQGKGFAVVAGEVRHLASRSAN
E.coli_O157_H7_Aer/353-406          TITSLINDIAFQTNILALNAAVEAARAGEQGKGFAVVAGEVRHLASRSAN
E.coli_O6_Aer/353-406               TITSLINDIAFQTNILALNAAVEAARAGEQGKGFAVVAGEVRHLASRSAN
E.coli_JM109_Tn1721_ORF1/354-4      KVLDVIRGIAEQTNLLALNAAIEAARAGEQGRGFAVVADEVRSLAARTQQ
L.interrogans_Aer/382-435           NIIGIITSISEKTNLLSLNASIESARAGEAGKGFSVVAEEISKLASQTRE
M.acetivorans_Hcp1/458-511          AVTGKIKSIADQTNLLALNAAIEAARAGEYGRGFAVVADEVRKLAADSRK
N.massei_TlpC/456-509               AVTGKIKSIADQTNLLALNAAIEAARAGEYGRGFAVVADEVRKLAADSRK
N.massei_Hcp/456-509                AVTGKIKSIADQTNLLALNAAIEAARAGEYGRGFAVVADEVRKLAADSRK
N.europaea/564-617                  DIISVIDGIAFQTNILALNAAVEAARAGEQGRGFAVVATEVRTLAQRSAA
P.aeruginosa_PAO1_Aer/353-406       GVVDVIKGIADQTNLLALNAAIEAARAGDQGRGFAVVADEVRSLAQRTAA
P.putida_PRS2000_Aer/353-406        EAAGHIRGIAEQTNLLALNAAIEAARAGEQGRGFAVVADEVRALADKTRQ
P.putida_KT2440_Aer-1/353-406       EAAGHIRGIAEQTNLLALNAAIEAARAGEQGRGFAVVADEVRALADKTRQ
P.putida_KT2440_Aer-2/353-406       GVVDVIKGIADQTNLLALNAAIEAARAGEHGRGFAVVADEVRQLAQRTAE
P.putida_KT2440_Aer-3/353-406       SVVDVITSIAEQTNLLALNAAIEAARAGEQGRGFAVVADEVRSLAQRTRA
P.resinovorans_CA10_pCAR1_ORF1      AAAGVIQAIADQTNLLALNAAIEAARAGEQGRGFAVVADEVRALAAKTRV
P.stutzeri/124-177                  AAAGVIQAIADQTNLLALNAAIEAARAGEQGRGFAVVADEVRALAAKTRV
P.syringae_DC3000_Aer1/353-406      SVVDVITSIAQQTNLLALNAAIEAARAGEQGRGFAVVADEVRSLASRTQT
P.syringae_DC3000_Aer2/353-406      GVVDVIKGIADQTNLLALNAAIEAARAGEHGRGFAVVADEVRQLAQRTAE
R.eutropha/372-425                  DIIGVIEGIAFQTNILALNAAVEAARAGEQGRGFAVVAGEVRSLAQRSAN
R.solanacearum_RSC03168_megap1      DIIGVIDSIAFQTNILALNAAVEAARAGEQGRGFAVVAGEVRALAQRSAS
R.solanacearum_RSC03711_megap1a     DIIGVIDSIAFQTNILALNAAVEAARAGEQGRGFAVVAGEVRALAQRSAS
R.meliloti_1021_HcpY/391-444        NIIGVIDDIAFQTNLLALNAGVEAARAGEAGKGFAVVAQEVRELAQRSAS
R.meliloti_RU11/391-444             NIIGVIDDIAFQTNLLALNAGVEAARAGEAGKGFAVVAQEVRELAQRSAS
S.enterica_Aer/353-406              TITTLINDIAFQTNILALNAAVEAARAGEQGKGFAVVAGEVRHLASRSAN
S.typhimurium_LT2_Aer/353-406       TITTLINDIAFQTNILALNAAVEAARAGEQGKGFAVVAGEVRHLASRSAN
S.oneidensis/347-400                QVLEVIRAIADQTNLLALNAAIEAARAGESGRGFAVVADEVRQLSSRTSQ
V.cholerae_VC0512/358-411           KVLEVIPSIADQTNLLALNAAIEAARAGESGRGFAVVADEVRGLAARTQQ
V.cholerae_VC0098/444-497           DIIGVIDGIAFQTNILALNAAVEAARAGEQGRGFSVVASEVRSLAQRSAN
Y.pestis_bv.Mediaevalis/382-43      DIISVIEGIAFQTNILALNAAVEAARAGEQGRGFAVVASEVRHLAQRSST
Y.pestis_bv.Orientalis/363-416      DIISVIEGIAFQTNILALNAAVEAARAGEQGRGFAVVASEVRHLAQRSST
                                           * *: :** *:*** :*:*****: *:**:*** *:  *:  :
```

Figure 3.3 Multiple alignment of the highly conserved domains of PAS-bearing taxis transducer proteins. (*) indicates entirely conserved columns (:) indicates columns where the residues have the same size and hydropathy. See Table 3.1. for full names of bacterial species used in this alignment.

```
A.vinelandii_AerPH/8-74          TQHEYELRDDQNLLSRTDLKGRITYAAPGFIEASGYSREELIGA---PHS
A.vinelandii_AerB/8-74           SGREYELQEDDFLISRTDLQGRITYANPAFVKVSGFPHEELMGA---PHN
A.salinestris_Aer5/8-74          TQHEYELRDDQNLLSRTDLNGRITYAAPGFIEASGYSREELIGA---PHS
A.tumefaciens_pTiSAKURA/10-77    ACAVLAALSKSQAMIEFDLTGRILTANDNFCRALGYELAEIIGK---HRS
A.tumefaciens_pTi_C58/15-82      ACAVLAALSKSQAMIEFDLSGRILTANDNFCRALGYELSEIIGK---HRS
A.tumefaciens_C58_atu3094/10-7   AVAVLAALSKSQAIIEFDLSGRILTANESFCRAIGYELAEIVGR---HRS
A.tumefaciens_C58_atu2618/10-7   NRNELDAISRSQAVIEFDLKGNILTANRNFCMALGYDLAEIVGK---HHR
A.fulgidus_TLPC2/64-131          VPELVRQIPKPAFVLFLNKDGIIEYINEYAAEVYGAEISEMIGRKP--SE
B.japonicum/8-74                 TDVEYPVSDETLIVSRTDLKGKLTYFNEDFLAAAGFTSAELMGQ---PHN
C.jejuni_CetBA/1-67              HSREIFLQEDSLITSKTDLKGKIVYANDDFLKYAGYTHGEVLNK---PHN
C.crescentus_HcpH/41-108         ASQKIDALDKSLAMIEFDVKGTILAANANFCKAMGYEAREIIGK---HHS
C.violaceum_Aer_HcpIV/10-76      GGREVQLAPHELIVTKTDPTGHITYANKVFERIAGYAEHQLLGQ---PHK
E.coli_K12_Aer/8-74              TQQNTPLADDTTLHSTTDLQSYITHANDTFVQVSGYTLQELQGQ---PHN
E.coli_O157_H7_Aer/8-74          TQQNTPLADDTTLHSTTDLQSYITHANDTFVQVSGFTLQELQGQ---PHN
E.coli_O6_Aer/8-74               TQQNTPLADDTTLHSTTDLQSYITHANDTFVQVSGYTLQELQGQ---PHN
E.coli_JM109_Tn1721_ORF1/8-74    TGRNLELPKDANILSTTSPQSHITYVNPDFIKISGFTEEELLGQ---PHN
L.interrogans_Aer/8-74           TSQEITIPINSVLISRTDMKGRISYVSQDFANISGFSEEEMLGE---PHN
H.acetivorans_Hcp1/33-103        VSSLIDNLPITVFRTSVDSSWAVRYISKNVDVLTGHTKHDFISQKI-SVS
M.mazei_TipC/31-101              TGWLIDNLPVTVFRVSNESSWPICYISKSVEVLTGYPAAEFLSPRL-SVS
M.mazei_Hcp/31-101               ISLLIDSLPVTVFRISNESSWAIHYIGKSVEQLTGYSKHDFITRKL-TVS
N.europaea/8-74                  TNVERENRDGEFLVSKTTAKGVITYINEPFIRHSGFTEQELVGQ---AHN
P.aeruginosa_PAO1_Aer/8-74       TQHERVTPAEQRLITTTNLKGIITYCNEAFIDISGFSREELHSA---PHN
P.putida_PRS2000_Aer/8-74        TPVEQRFPSGQRLISATDTASLITYCNPEFAAISGYSDAELIGS---PHN
P.putida_KT2440_Aer-1/8-74       TPVEQRFPPHQRLISATDTASLITYCNPEFAAISGYSEAELIGS---PHN
P.putida_KT2440_Aer-2/8-74       TQRERTFPAQQRLISTTNAKGVITYCNDAFIEISGFTREELTGA---PHN
P.putida_KT2440_Aer-3/8-74       TEHERTFPGDQRLISTTDLDSRITYCNDAFVAISGFTYDELVGQ---PHN
P.resinovorans_CA10_pCAR1_ORF1   SAIYRAAVDAAAIFSETDKSGIITYVNEQFCDISGYSAQELIGQ---NHR
P.syringae_DC3000_Aer1/8-74      TQTERTFPASERLISTTDLNSQITYCNDAFVTLSGFTREELIGQ---PHN
P.syringae_DC3000_Aer2/8-74      TQRERTFPAEQRLISTTDTRGTITYCNDAFVDISGYSEAELLGA---AHN
R.eutropha/11-77                 TQNEYKLSPDDYLISRTDLKGRITFANRTFVEASGFSAQELLGA---AHN
R.solanacearum_RSO63168_megap1   TQREYEFPDNATLHSTTDTQSYIAYANAAFVDVSGFSREEIEGQ---PHN
R.solanacearum_RSO3711_megap1a   TQREYEFPDNATLHSTTDTQSYIAYANAAFVDVSGFSREEIEGQ---PHN
R.meliloti_1021_HcpY/10-77       APQIIDALSKSQAIIQFDLAGNVLDANENFCKALGYSLQEIVGQ---HHR
R.meliloti_RU11/10-77            ARQIIDALSKSQAIIQFDLAGNVLDANENFCKALGTSLQEIVGQ---HHR
S.enterica_Aer/8-74              SQLNTPLDDDTTLHSTTDLESYITHANDTFVQVSGYQLNELLAP---PHN
S.typhimurium_LT2_Aer/8-74       SQLNTPLDDDTTLHSTTDLESYITHANDTFVQVSGYQLNELLAQ---PHN
S.oneidensis/8-72                GEK--HLSENAILLSTTDLKGNIKYVNQTFSQISEYSVAELQGS---PHN
V.cholerae_VCOS12/12-78          TGHNIELSSSTNILSTTTPDSHITYVNPDFLKISGFAEEELIGQ---PHN
V.cholerae_VCO098/65-130         NQAFVQVIREHLALLECEPNGTICYASDAFAHLCPVSAEAMVGAD--FAM
Y.pestis_bv.Mediaevalis/27-93    SRQEYPIERDITLQSTTDIHGNIAYANAAFVRASGFEYQELQGQ---PHN
Y.pestis_bv.Orientalis/8-74      SRQEYPIERDITLQSTTDIHGNIAYANAAFVRASGFEYQELQGQ---PHN
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Figure 3.4 Multiple alignment of the PAS domains from PAS-bearing taxis transducer proteins. (:) Indicates columns where the residues have the same size and hydropathy (.) indicates columns that have semi-conserved residues. See Table 3.1 for full names of bacterial species used in this alignment. For the proteins which contain multiple PAS domains only the first PAS domain was used in this alignment.

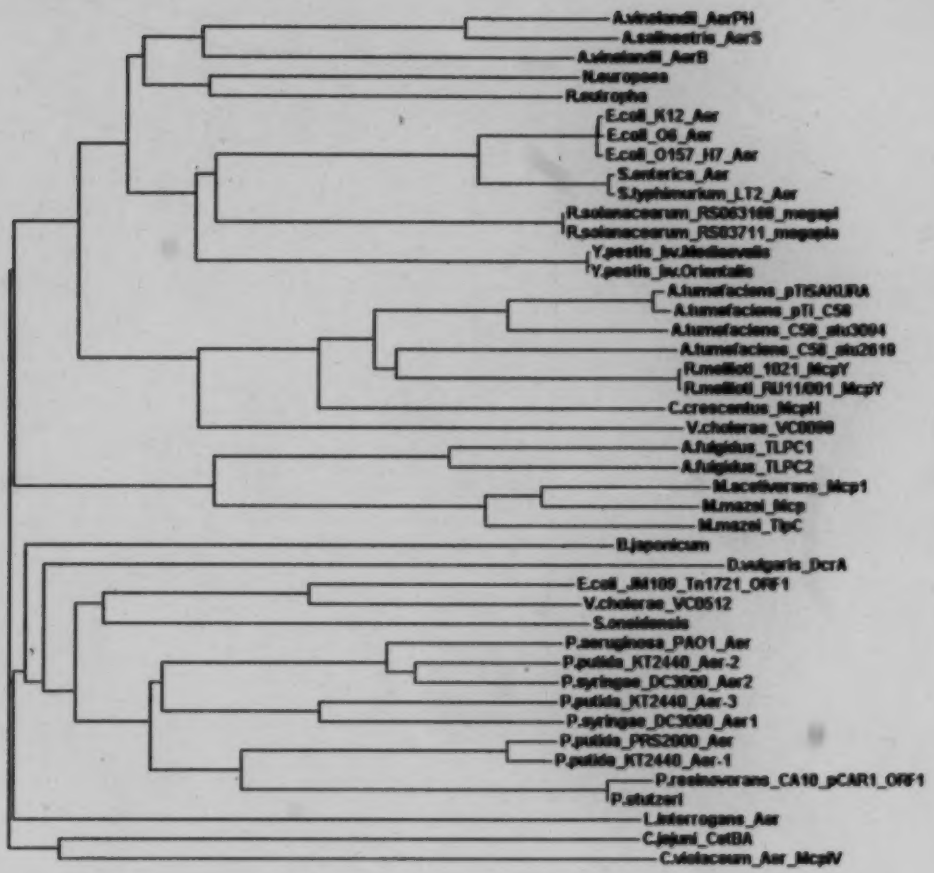

Figure 3.5 Phylogenetic tree of PAS-bearing taxis transducer proteins. Tree was constructed by the neighbour-joining method using ClustalW. All gapped columns in the multiple alignment were ignored for the computation of this tree.

same or closely related species. One exception is the *E. coli* transducer encoded on Tn1721, which groups with a *V. cholerae* transducer.

3.2 Construction of transducer null mutants

To examine the role of that the taxis transducer proteins play in *A. vinelandii* motility the transducer genes were mutagenized by insertion of antibiotic resistance cassettes. For complete mutational strategies and cloning reactions see Figure 3.6 and Table 2.1. A 3,254-bp PCR fragment bearing *aerP* and *aerH* was cloned into pCR®2.1-TOPO to yield pHW1. The *aerP* and *aerH* genes were disrupted by inserting the ΩSm^R cassette from pHP45Ω between two *Sma*1 sites on pHW1. This digestion and subsequent ligation resulted in the liberation of a 1,368-bp fragment of *aerP* and *aerH* and yielded the plasmid pHW2. The gene *aerH* was disrupted alone by inserting the ΩSm^R cassette into a unique *Nde*1 restriction site in pHW1 to yield pHW3.

The gene *aerP* was disrupted by insertion of the Km^R cassette from p34S-Km3 into a unique *Sma*1 site of the plasmid pHW5 that contains a 1,020-bp fragment of *aerP*. The Km^R cassette used contains weak terminators and the plasmids generated from this cloning reaction were screened and sequenced to confirm that the Km^R cassette was in the non-polar orientation.

A 2,119-bp PCR product bearing *aerB* was cloned into pDrive to yield pHW6. The gene *aerB* was disrupted by inserting the ΩTc^R cassette from pHP45Ω-Tc into a unique *Nco*1 restriction site in pHW6 to yield pHW7. Previous researchers have shown that the terminators used in the Ω series of cassettes are weak and do not result in polar

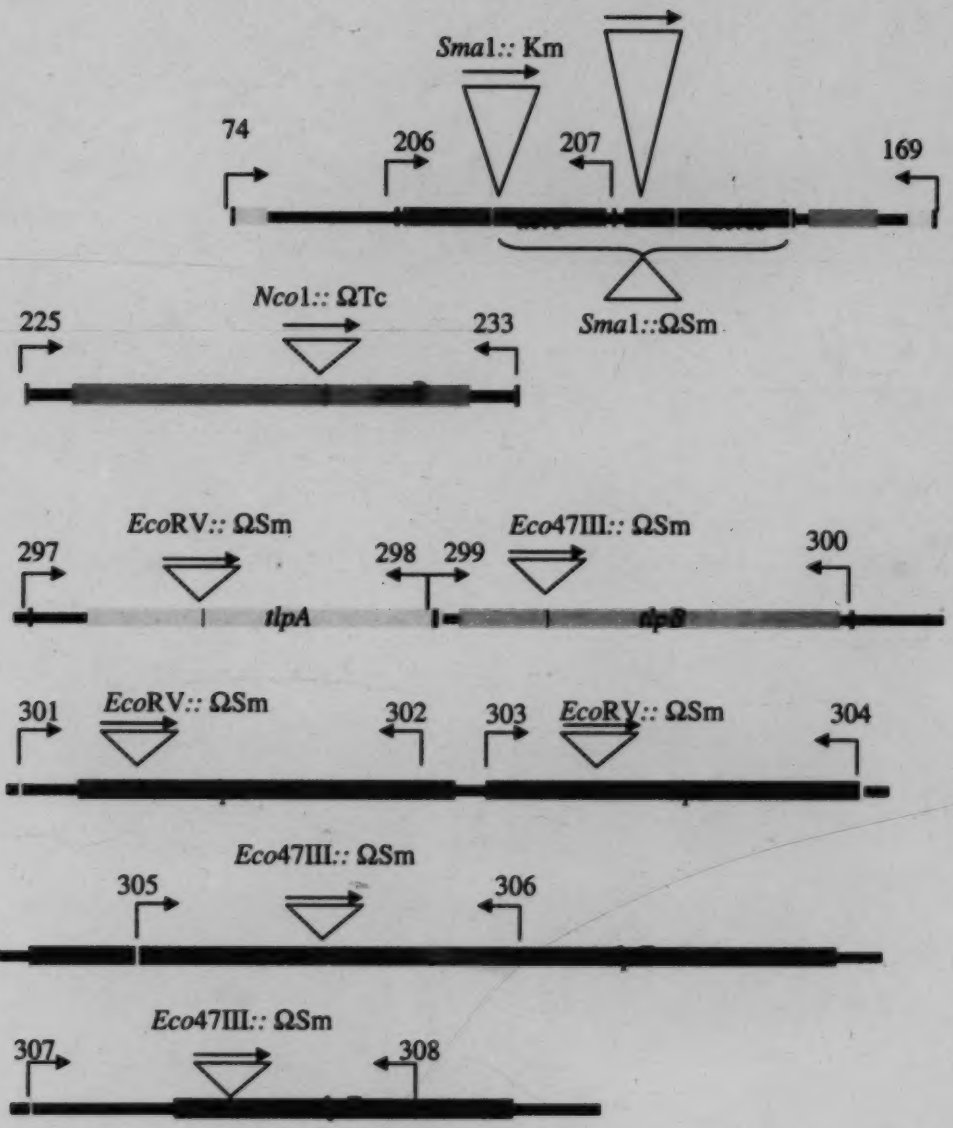

Figure 3.6 Mutation strategy for disruption of taxis transducer proteins in *A. vinlandii*. Primers used to amplify specific genes are shown. Triangles indicate sites where cassettes were inserted into the gene and arrows above triangles indicate direction of antibiotic resistance gene in cassette. See text and Table 2.1 and Table 2.2 for details.

mutations when inserted directionally into the gene being disrupted (Mouncey *et al.*, 1995; Vazquez *et al.*, 1999). Sequencing was used to confirm that the cassette was inserted in the non-polar orientation. The six classical transducer genes were disrupted using a similar approach. The genes *tlpA, tlpB, tlpC, tlpD, tlpE* and *tlpF* were amplified using gene specific primers and the products were cloned into pGEM®-T Easy to yield pHW14-19, respectively. The genes were then disrupted by inserting the ΩSm^R cassette into unique *Eco*RV or *Eco*47III sites (Figure 3.6). The resulting transformants were screened by restriction digest analysis and sequencing to confirm that the ΩSm^R cassette was inserted in the same direction as the gene to prevent any polar effects.

The gene-disrupted plasmid constructs were then transformed into motile, naturally-competent *A. vinelandii* UW (for a complete list of mutants created and their names see Table 2.1). For the construction of the triple mutant (designated *aerB,PH)* genomic DNA from the *aerB* mutant was transformed into motile, naturally-competent Δ*aerPH* mutants. Resulting transformants, resistant to the appropriate antibiotic marker, were screened for the occurrence of double crossover events and maintained on selective media. In most cases several transformant colonies were examined on soft agar plates to ensure that all transformants behaved identically. In all cases the phenotypes were consistent among the antibiotic-resistant transformants.

3.3 Motile behaviour of *aerB*, Δ*aerPH*, *aerP*, *aerH*, and *aerB,PH* mutants

3.3.1 Soft agar plate assays

The first challenge in studying the motility of *A. vinelandii* was developing a suitable assay. The soft agar tube assays used by Haniline *et al.* (1991) were difficult to reproduce and direct microscopic observation was also problematic. Eventually the soft agar plate assay, first developed by J. Adler, was adapted for *A. vinelandii*. Initial experiments were set up using 1-3 mM of various carbohydrate and organic acid carbon sources. These experiments were unsuccessful, there were no swimming halos produced. The concentration of chemicals tested was increased to 10 mM and higher and swimming halos were observed. Numerous carbon compounds, listed in Table 3.2, were tested for their ability to produce halos. Any carbon source that could also be used as an energy source was able to elicit the swimming response, provided it was supplied in a sufficient concentration, generally a minimum of 10 mM. The notable exceptions to this were citrate and oxaloacetate, which did not produce swimming halos. Sugars and organic acids were common test substrates in the soft agar plates and one consistent observation was the change in halo appearance on these two substrate types. Distinct rings with a very defined edge were always observed on the organic acids whereas the edge of the sugar halos was rougher and less defined. The halos were often very faint and capturing images became difficult, even when tetrazolium dye was used.

The *aer* mutants were examined for their ability to swim in soft agar media containing various carbon sources. No difference in performance was observed for the Δ*aerPH*, *aerP* or *aerH* mutants, compared to the wild type. The *aerB* mutant, however, consistently performed differently from the wild type strain on all carbon compounds tested. Figure 3.7A and B shows the response of the various energy taxis mutants on 10 mM fumarate plates. The *aerB* and *aerB,PH* swimming halos are

Table 3.2 Carbon compounds tested as motility substrates

Chemical type	Name	Swimming halo	Growth substrate*
Aldohexose	glucose	+	+
	fructose	+	+
	galactose	+	+
Deoxy sugar	rhamnose	+	+
Disaccharide	mannose	-	-
	maltose	+	+
	sucrose	+	+
Trisaccharid	raffinose	+	+
	mellibiose	+	+
Sugar alcohols	sorbitol	+	+
	mannitol	+	+
	arabitol	+	+
Organic acids	acetate	+	+
(TCA	citrate	-	+
cycle	isocitrate	+	+
intermediates)	succinate	+	+
	fumarate	+	+
	malate	+	+
	oxaloacetate	-	+
	glyoxylate	-	NL
	alpha ketoglutarate	+	NL
Other	betahydroxy butyrate	+	+
	glycerol	+	+
	ethanol	+	+
Aromatic	phenol	+	+
	resorcinol	+	+
	benzoate	+	+
	3,4 (o) dihydroxy benzoic acid	+	NL
	2,5 (p) dihydroxybenzoic acid	-	NL
	3,5 (m) diaminobenzoic acid	+	NL

* As identified by Thompson & Skerman (1979); NL not listed

Figure 3.7 Motility of *A. vinelandii* UW and energy taxis transducer mutants on soft agar media. (A) 10 mM fumarate 0.275% agar (clockwise from top left: UW, Δ*aerPH-*, *aerH-*, *aerP-*). (B) 10 mM fumarate 0.275% agar (clockwise from top left UW, *aerB-*, *aerB,PH-*, *aerB,PH-*). (C) 10 mM glucose 0.5% agar (clockwise from top left: UW, Δ*aerPH-*, *aerH-*, *aerP-*). (D) 10 mM glucose 0.5% agar (clockwise from top left UW, *aerB-*, *aerB,PH-*, *aerB,PH-*).

noticeably smaller and more defined at the border then the wild type strain. This effect is not observed in the Δ*aerPH*, *aerH* or *aerP* mutants, these mutants appear to swim exactly like the wild type cells. The motility of the *aerB* and *aerB,PH* mutants is noticeably impaired on glucose containing soft agar plates when the agar concentration is increased to 0.5% (Figure 3.7C and D).

Soft agar media containing a variety of carbon sources was prepared and inoculated with UW, Δ*aerPH*, *aerH* and *aerB*. The diameter of the swimming halos of the different strains was measured after 24 hours incubation (Figure 3.8). A clear reduction in halo diameter was observed for the *aerB* strain compared to all other strains tested. A lag in motility for *aerB* was most noticeable when glucose was the sole carbon source, but was also evident when organic acids or sugar alcohols were the sole carbon source. No difference was observed in the diameter of the halos on glycerol medium, however the *aerB* mutants produced more defined halo edges. The altered appearance on the *aerB* mutant also was a consistent observation on all carbon sources tested.

3.3.2 Aerotaxis assays

The *A. vinelandii* wild-type and *aer* mutant strains were examined for their ability to move in response to oxygen gradients. Optically flat capillaries were used to examine the response of the cells towards oxygen. The wild type cells clustered at the air-liquid interface and exhibited a highly motile behaviour within this microenvironment. Motility was noticeably reduced the further the cells moved away from the air interface. At a certain distance away from the air interface the cell's ceased motility altogether. As with

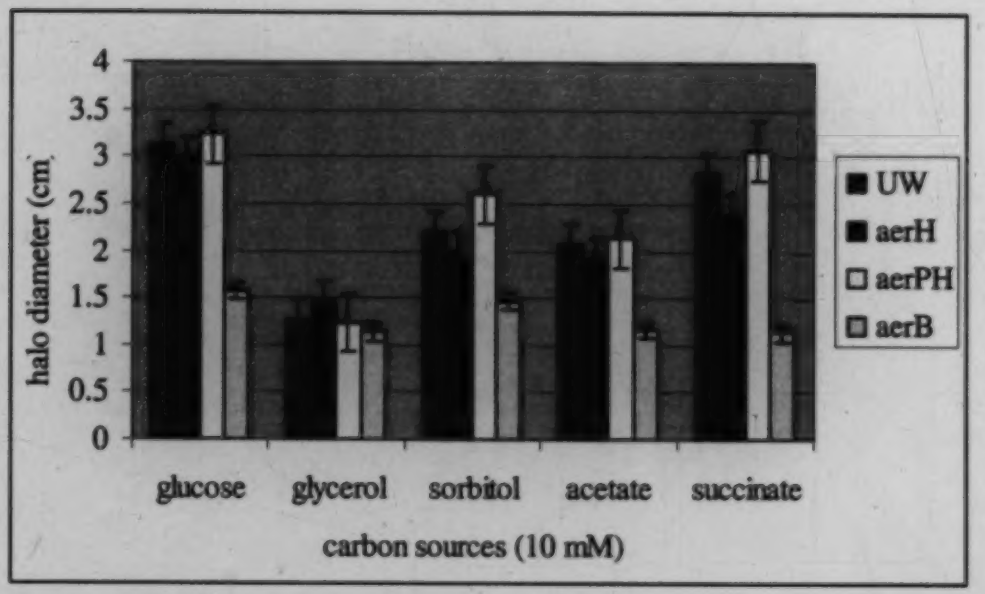

Figure 3.8 Swimming halo diameter of various *A. vinelandii* energy taxis transducer mutants on soft agar media after 24 hours. Triplicate data was used to calculate the mean halo diameter and standard error.

the soft agar plate assay the Δ*aerPH* mutants behaved identical to the wild type UW cells in these capillaries. The *aerB* and *aerB,PH* mutants, over time, formed a distinct band away from the air interface. Cells within this band were non-motile and cells on the distant side of the band from the air interface were also non-motile.

3.4 Motile behaviour of *tlpA*, *tlpB*, *tlpC*, *tlpD*, *tlpE* and *tlpF* mutants

The *tlp* mutants were examined for their ability to swim in several different soft agar media. Six carbon sources, present at 20 mM concentrations, were tested as motility substrates and the radius of the swimming halos were measured after 32 hours (Figure 3.9). The mutant *tlpB* had a significantly reduced motility on all compounds tested. On the organic acids, ammonium acetate, sodium acetate, and succinate, the *tlpC* mutant also appeared to have a reduced motility (Figure 3.9D, E and F). Similarly the *tlpD* mutant appeared to have a reduced motility on glycerol (Figure 3.9B). The *tlpA*, *tlpE* and *tlpF* mutants did not appear to have a reduced motility on any of the carbon compounds compared to the wild type, however on the sodium acetate plates these mutants, along with *tlpD*, appeared to have swam much further than UW (Figure 3.9E). The *aerB* mutant was also tested and as expected it had an altered motility on all compounds tested. The altered motility is not always represented well in the halo radius measurement, rather the *aerB* mutant produced more sharply, defined halos then the wild type cells.

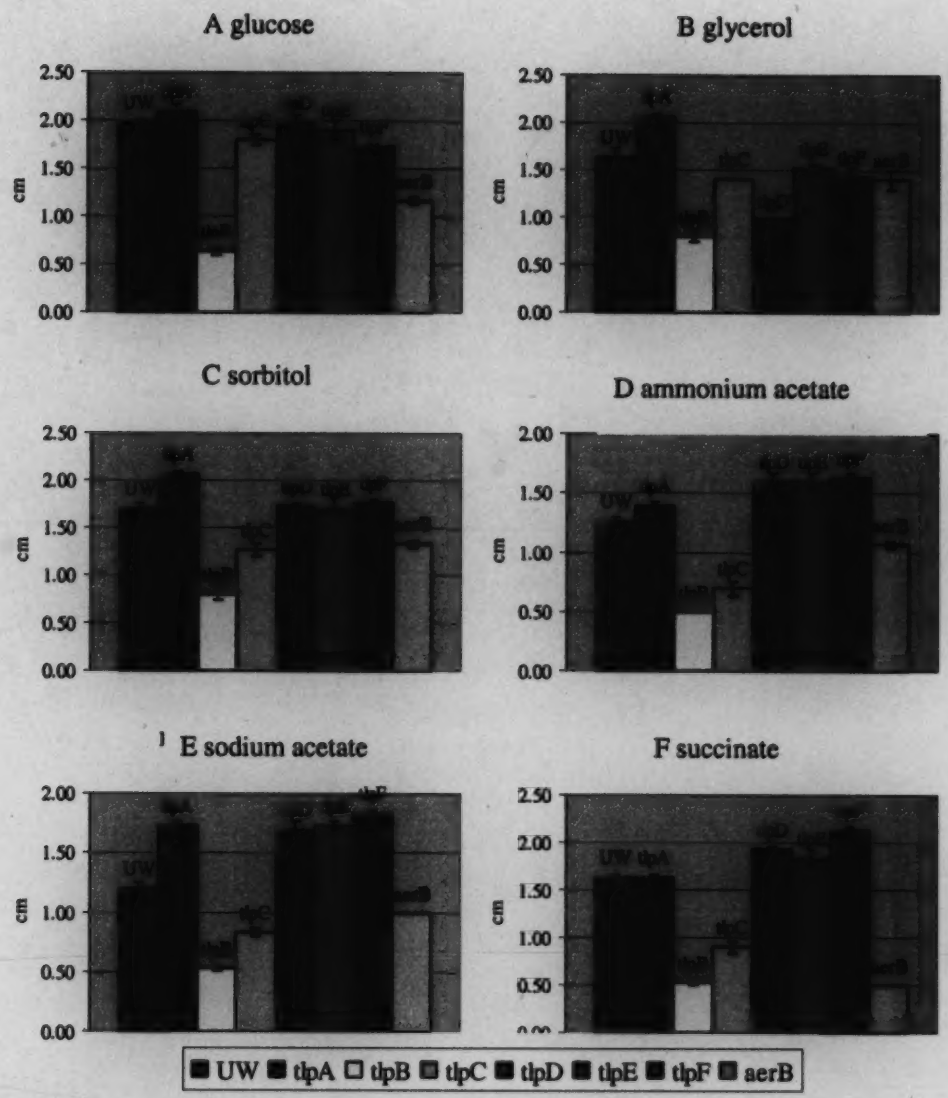

Figure 3.9 Swimming halo radius of various *A. vinelandii* transducer mutants on soft agar media. Media contains 20 mM carbon source. Error bars are calculated from triplicate data sets from a single experiment.

3.5 Pattern formation by motile *A. vinelandii*

3.5.1 Discovery of pattern formation and autoaggregation behaviour

During the course of this project one of the goals was to investigate the chemotactic behaviour of *A. vinelandii* towards amino acids. Motility experiments using soft agar plates, prepared to contain varying concentrations of amino acids as the sole carbon source, were performed. No evidence of motility or growth was observed in these experiments. This result was not unexpected, as *A. vinelandii* cannot use amino acids as a sole carbon source. The experiments were modified and glucose was added to the plates to serve as a sole carbon source. Various amino acids were added to the glucose media prior to autoclaving including: asparagine, glutamine, histidine, lysine, proline, and arginine. Surprisingly the cells that had been inoculated in the asparagine medium formed autoaggregates in a distinct pattern formation (Figure 3.10). No other amino acid tested was able to result in pattern formation. Furthermore the autoaggregation was only observed when the asparagine was added prior to autoclaving the medium, suggesting that a breakdown product of asparagine is ultimately responsible for pattern formation. Lastly it was also observed that only very high-density inocula were able to autoaggregate and develop into the patterns.

These are the first descriptions of this aggregation behaviour in *A. vinelandii* and the specific conditions used to generate the patterns have never been described for any prokaryotic organism. The following section will describe how different variables can be manipulated to generate different patterns. Throughout this work the term autoaggregate

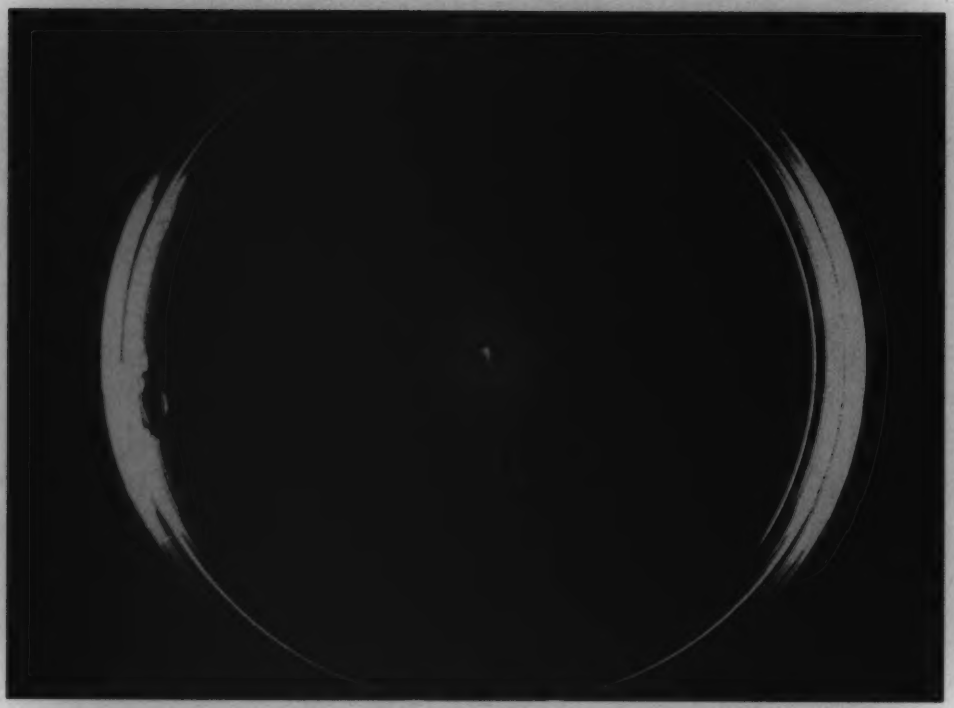

Figure 3.10 Chemotactic autoaggregates formed by *A. vinelandii* UW on medium containing 2.5 mM autoclaved asparagine, 30 mM glucose and 0.3% agar.

will be used to describe the macroscopic "spots" and "stripes" that the bacteria swim to form.

3.5.2 Effect of asparagine concentration on pattern formation.

Numerous experiments were performed and have demonstrated that the concentration of asparagine is a critical factor determining whether the cells will autoaggregate and form patterns. When describing the concentration of asparagine used in this study it is the initial concentration of asparagine, prior to autoclaving that is being described. This is done with the understanding that the composition of the medium is altered during the sterilization process. Figure 3.11 shows the effect of altering asparagine concentration on pattern formation. No autoaggregation was observed at 2.0 or 3.0 mM asparagine but patterns were present at 2.5 mM asparagine (Figure 3.11B). Altering the asparagine concentration between the range of 2.2 mM and 2.6 mM did not appear to affect the patterns significantly (Figure 3.12).

3.5.3 Effect of acrylamide and other compounds on pattern formation

Several chemicals similar in structure to asparagine were tested for their ability to yield patterns. The chemicals tested were L-asparagine, D-asparagine, Nα-acetyl-L-asparagine, succinamic acid and succinamide (structures shown in Figure 3.13). All the chemicals, once autoclaved, resulted in pattern formation. As with L-asparagine the

Figure 3.11 Loss of autoaggregation behaviour at high and low concentraions of autoclaved asparagine. The medium contains 30 mM glucose, 0.3% agar and (A) 2.0 mM asparagine, (B) 2.5 mM asparagine, or (C) 3.0 mM asparagine.

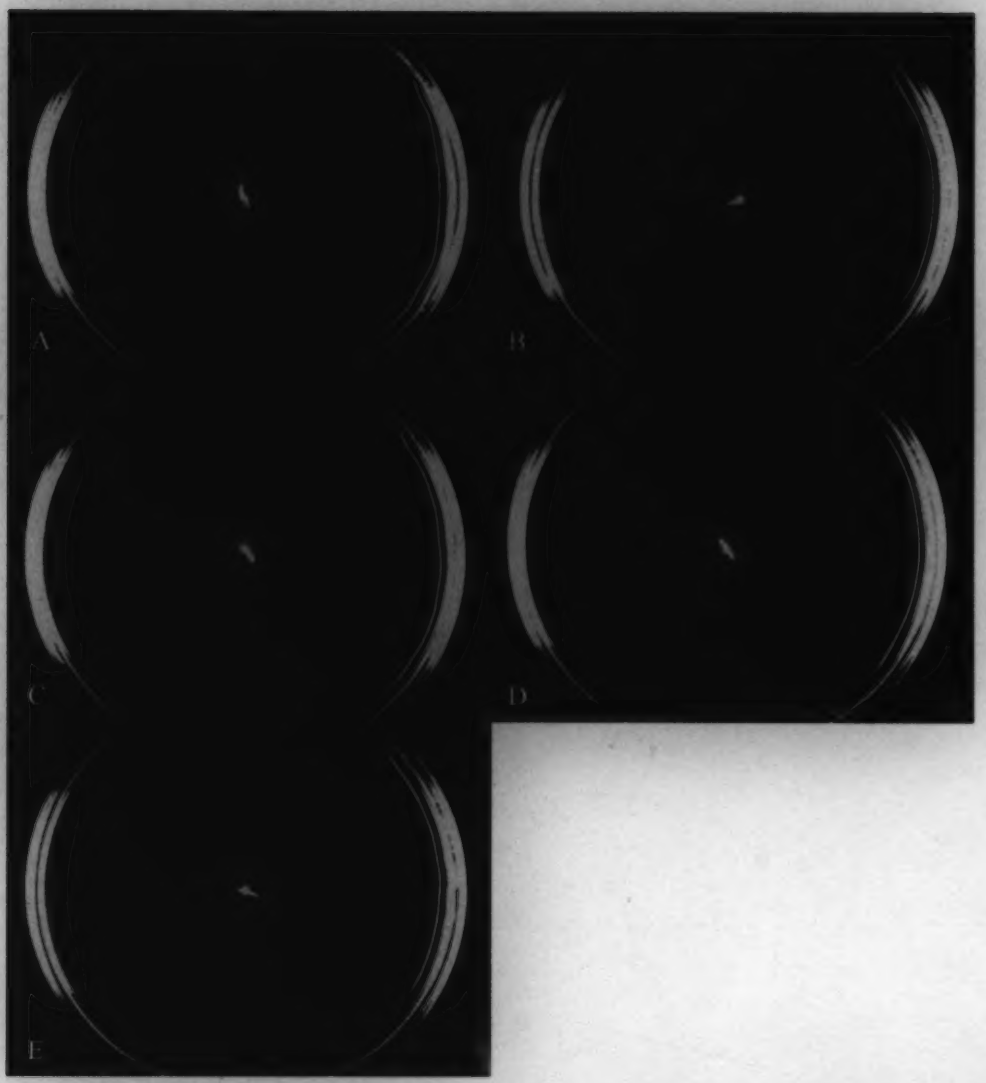

Figure 3.12 Autoaggregation occurs at initial asparagine concentrations of 2.2-2.6 mM. The medium contains 30 mM glucose, 0.3% agar and (A) 2.2 mM asparagine, (B) 2.3 mM asparagine, (C) 2.4 mM asparagine, (D) 2.5 mM asparagine, or (E) 2.6 mM asparagine.

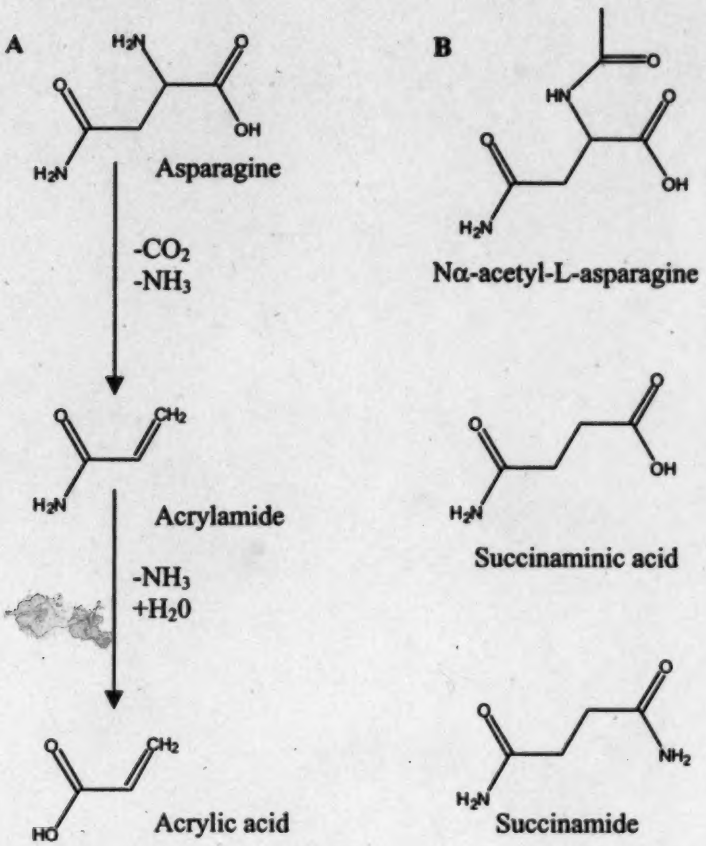

Figure 3.13 Breakdown of asparagine to acrylic acid and structures of asparagine-like compounds as substrates for autoaggregation. (A) Proposed breakdown scheme of asparagine to acrylamide to acrylic acid by the Maillard reaction; adapted from Mottram *et al.* (2002). This reaction is believed to occur during the sterilization process such that one molecule of asparagine is broken down to one molecule acrylic acid, one molecule CO_2, and two molecules NH_3. (B) Structure of the three asparagine-like compounds which, once autoclaved, yield autoaggregation.

concentration of these chemicals was important for pattern formation, with 2.5 mM yielding patterns.

The observation that all asparagine-like chemicals must be autoclaved with the medium in order to yield patterns suggested that a breakdown product of asparagine was responsible for autoaggregation. Acrylamide, a known breakdown product of asparagine (Mottram *et al.*, 2002), was investigated for its ability to yield patterns. A filter-sterilized solution of acrylamide was added to sterile medium to a final concentration of 2.5 mM. This medium was inoculated with motile cells and the resulting patterns are shown in Figure 3.14. Autoaggregates developed in the acrylamide medium, but the arrangement of the aggregates was not the same symmetrical and ordered pattern that was found in the autoclaved asparagine medium.

Medium containing 2.5 mM asparagine was autoclaved and analysed for the presence of acrylamide or acrylic acid using chromatography. No acrylamide was detected in the autoclaved sample. Approximately 2.5 mM acrylic acid was detected in the autoclaved sample (M. Haveroen, personal communication). A non-autoclaved control sample was also tested and no acrylamide or acrylic acid was detected. The proposed breakdown scheme of asparagine to acrylamide and finally to acrylic acid is shown in Figure 3.13A.

3.5.4 Pattern development over time

Patterns generally became visible after 12-18 hours of incubation and continued to expand until the entire plate surface was covered. The patterns were macroscopic and

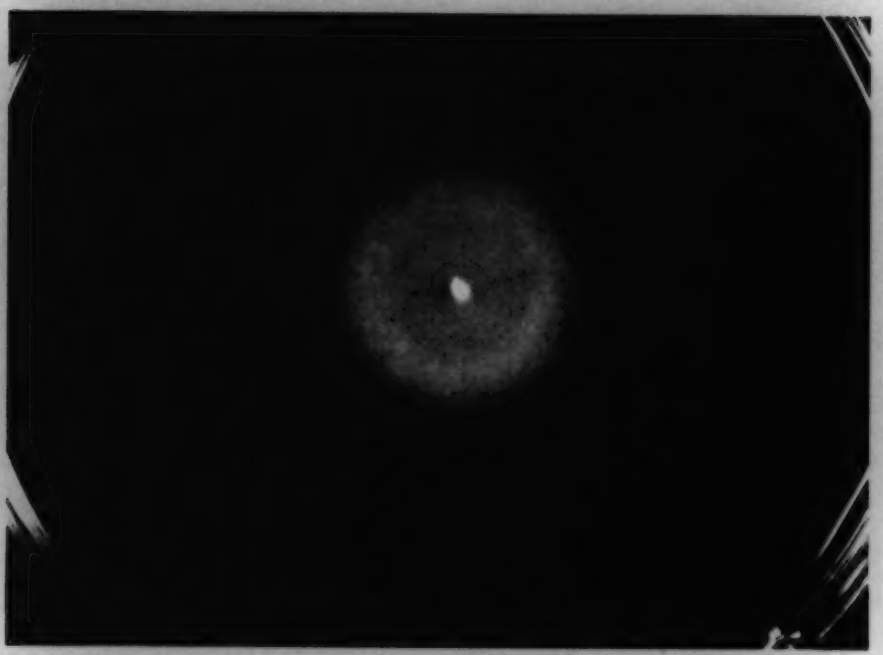

Figure 3.14 Chemotactic autoaggregates formed in soft agar containing acrylamide and glucose. Medium contains 2.5 mM acrylamide, 30 mM glucose, 0.3 % agar. Paattern produced after 46 hours incubation.

developed over time (Figure 3.15). The autoaggregates, once formed, were stationary and continued to develop outwardly in a circular fashion. Figure 3.15A shows patterns formed on 2.5 mM asparagine, 0.5 mM acrylamide and 90 mM glucose medium. In this medium the radius of the pattern increases from 13.5, 20.5 to 26 mm after 18, 22, and 26 hours of inoculation, a speed of 1.56 mm/h. Figure 3.15B shows a different pattern produced on 2.5 mM asparagine, 2.5 mM acrylamide and 90 mM glucose. The higher acrylamide concentration affects the appearance of the patterns and the radius of the pattern increases from 12.5, 17.5, to 21.5 mm after 18, 22, and 26 hours after inoculation, a speed of 1.125 mm/hour.

3.5.5 Effect of agar concentration on pattern formation

The concentration of agar in the soft agar media had a major effect on the development of the patterns. As agar concentration was increased the diffusion of chemicals through the medium became more limited. In very low concentrations of agar, around 0.2%, the autoaggregates appeared elongated and form patterns that lacked the ordered structure seen in other patterns (Figure 3.16A and B). At concentrations between 0.24-0.28% agar the autoaggregates were arranged in a more ordered pattern, where the aggregates tended to develop equidistant from one another (Figure 3.16C, D and E). As the agar concentration was increased to 0.3% the patterns did not develop regularly and the borders of the patterns did not resemble the circular patterns observed at lower agar concentrations (Figure 3.16F).

Figure 3.15 Pattern development over time. (A) 2.5 mM asparagine (autoclaved), 0.5 mM acrylamide, 90 mM glucose 0.25% agar. (B) 2.5 mM asparagine (autoclaved), 2.5 mM acrylamide, 90 mM glucose 0.25% agar. From left panel to right panel 18, 22 and 26 hours after inoculation.

Figure 3.16 Effect of agar concentration on pattern formation. The medium contained 100 mM glucose, 2.5 mM asparagine and (A) 0.20% agar, (B) 0.22% agar, (C) 0.24% agar, (D) 0.26% agar, (E) 0.28% agar, or (F) 0.30% agar.

In some cases the lower agar concentrations induced the bacteria to form spots which then continued to move outward, such that the spots took on a teardrop-like appearance, yielding striped patterns. The development of these striped patterns over time is shown in Figure 3.17. The original position of the autoaggregation remained stationary, yet the cells continued to move together away from this point. The borders of the autoaggregates appeared sharply defined. New aggregates formed beyond these stripes as other cells continues to swim in a radial fashion.

3.5.6 Patterns formation from adjacent inoculation points

When the cells were inoculated at a single point in the medium the patterns developed outwardly in a radial fashion (Figure 3.18A). However when two adjacent points in the soft agar medium were inoculated the patterns developed asymmetrically (Figure 3.18B). In patterns generated with autoclaved asparagine the aggregates continued to form outwardly from the inoculation point, but stopped abruptly at the interface between the two points (Figure 3.18B). This cessation of autoaggregation between inoculation points was reduced when acrylamide is added to the medium (Figure 3.18D). As the ratio of acrylamide to asparagine was increased the aggregates continued to develop between the inoculation points. When the plates contained only acrylamide and no autoclaved asparagine, there was no loss of aggregation between the inoculation points (Figure 3.18F).

The inability of the autoaggregates to form between the adjacent inoculation points was most dramatic as the patterns developed over time. At 22 hours after inoculation it

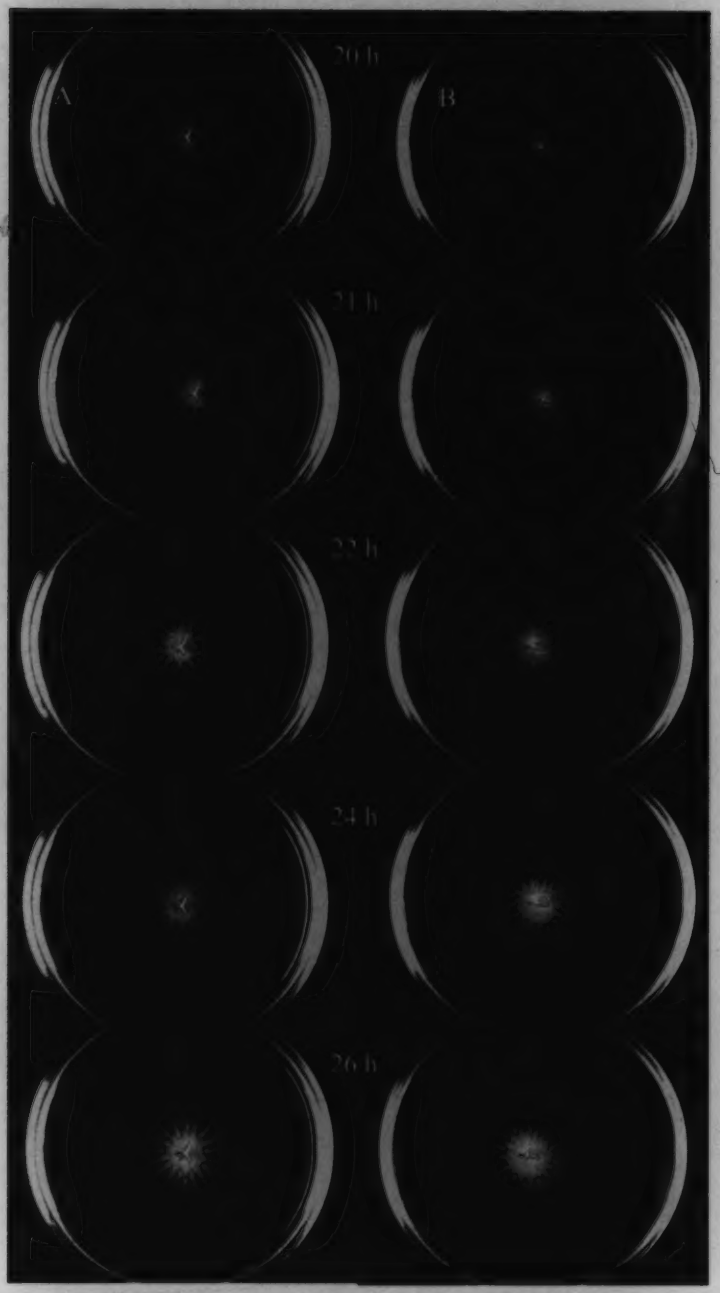

Figure 3.17 Development of striped patterns over time. The medium contained 90 mM glucose, 2.5 mM asparagine and (A) 0.2% agar, or (B) 0.225% agar.

Figure 3.18 Comparison of single and neighbouring patterns produced with increasing acrylamide concentrations. The medium contained: (A,B) 2.5 mM asparagine, 0.5 mM acrylamide, 90 mM glucose, 0.275% agar; (C,D) 2.5 mM asparagine, 2.5 mM acrylamide, 90 mM glucose, 0.275% agar; (E,F) 0.0 mM asparagine, 3.0 mM acrylamide, 90 mM glucose, 0.275% agar.

became apparent that the next row of autoaggregates did not form at the between the two points (Figure 3.19A). At 46 hours the autoaggregates continued to develop radially to the edge of the Petri plate, but continued to be repelled by the adjacent inoculation point (Figure 3.19B).

3.5.7 Generation of patterns around chemical soaked discs

In order to investigate the effects that the concentration of specific chemicals has on pattern formation a novel diffusion-based assay was developed. Highly absorbent paper discs, which had been saturated with the chemical of interest, were introduced aseptically to the surface of a soft agar plate. The cells were inoculated adjacent to the disc and the plates were examined the next day. Ammonium, another breakdown product of asparagine (Figure 3.13), was used in these assays. Figure 3.20 shows the results when motile *A. vinelandii* UW was inoculated near a disc soaked in 100 mM ammonium chloride. Autoaggregates formed on these plates at a specific distance form the disc. The cells that had moved too far away from the disc did not aggregate; similarly the cells too close to the disc did not form aggregates. The aggregates themselves changed appearance depending on the distance from the disc. The aggregates were longer and developed a more striped appearance the further away from the disc they formed.

Different patterns were generated when the concentration of ammonium chloride in the discs was altered (Figure 3.21). At 0, 1 and 5 mM ammonium chloride the cells swam outward from the inoculation point, but never aggregated (Figure 3.21A, B and C). When the ammonium chloride concentration was increased to 10 mM the aggregates

Figure 3.19 Development of patterns formed from adjacent inoculation points over time. The medium contained 90 mM glucose, 2.5 mM asparagine, 0.275% agar incubated for (A) 22 hours, or (B) 46 hours.

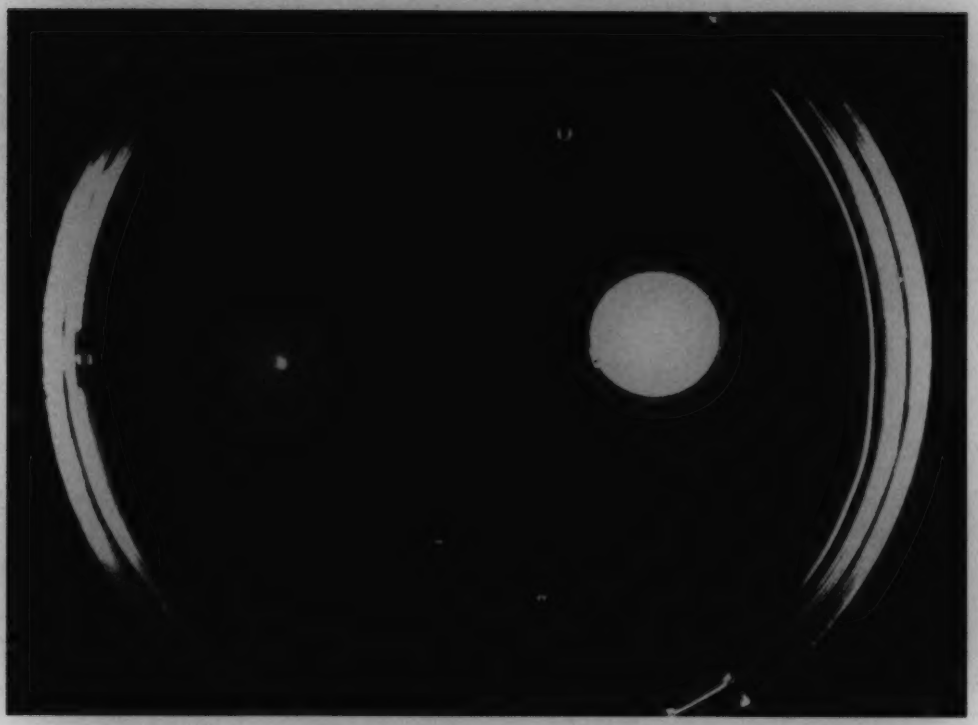

Figure 3.20 Chemotactic patterns formed by *A. vinelandii* UW around disc soaked in 100 mM ammonium chloride. The medium contains 100 mM glucose and 0.275% agar.

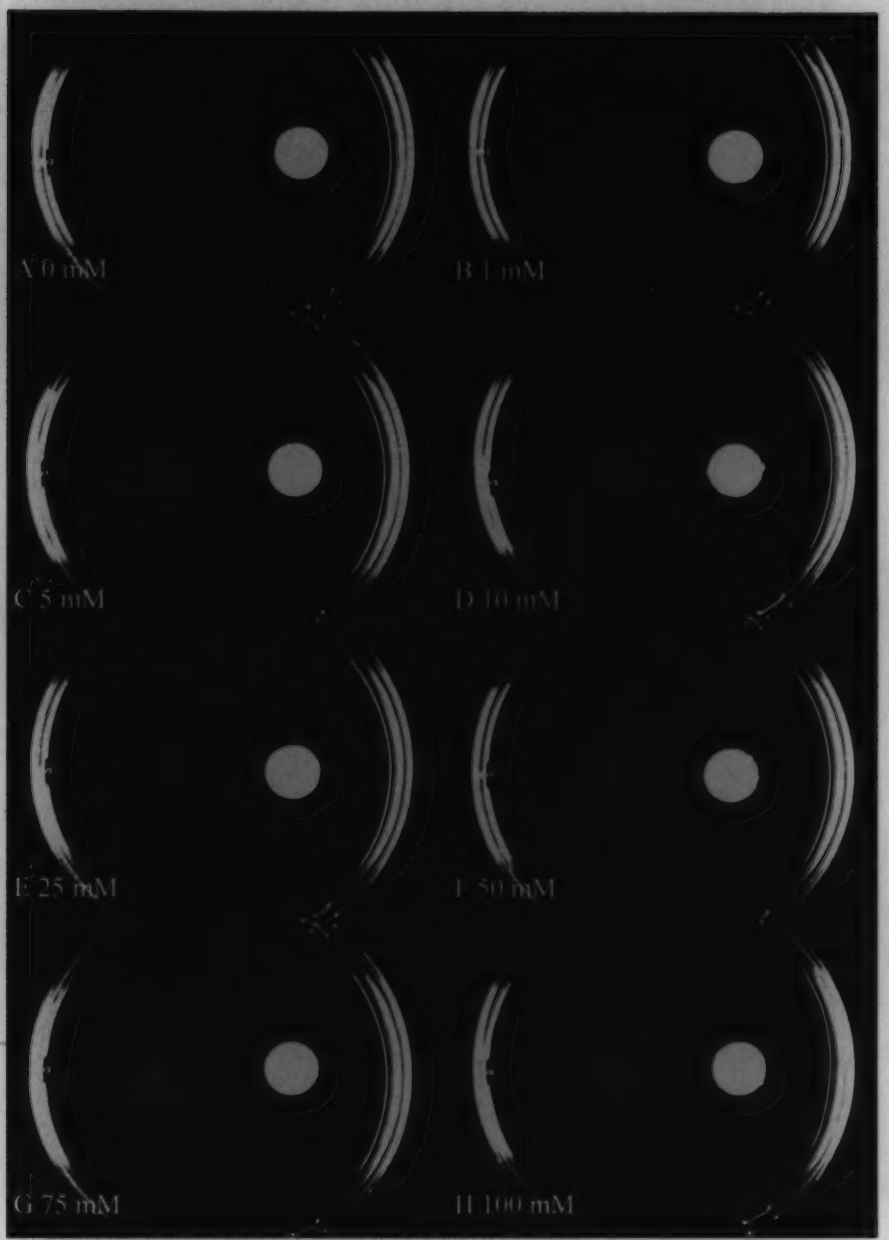

Figure 3.21 Autoaggregation of *A. vinelandii* UW around discs soaked in varying concentrations of ammonium chloride.

formed near the disc (Figure 3.21D). As the concentration of ammonium chloride was increased the aggregates continue to form at a distance from the disc, forming further away from the disc as the ammonium chloride concentration was increased (Figure 3.21 E, F, G and H).

Aggregation was not observed in media containing only ammonium and glucose. Various concentrations of ammonia (0.5-9.0 mM) were tested and no aggregation was observed. Reproducible autoaggregation of cells in response to ammonium was only observed in the soaked disk assays.

3.5.8 Patterns formed by mutant strains

The mutant strains were examined for their ability to form the patterns, in order to identify the taxis transducers essential for pattern formation. The strains were tested for their ability to autoaggregate and form patterns in response to discs soaked in 10 mM ammonium chloride (Figure 3.22). Once again the *aerB* and *aerB,PH* mutants were impaired in motility and did not form the aggregates like UW. No behavioural difference was found for the Δ*aerPH* mutant. The transducer knock out mutants: *tlpD*, *tlpE* and *tlpF* were capable of producing the patterns, whereas the *tlpA*, *tlpB* and *tlpC* mutants were not able to form the patterns.

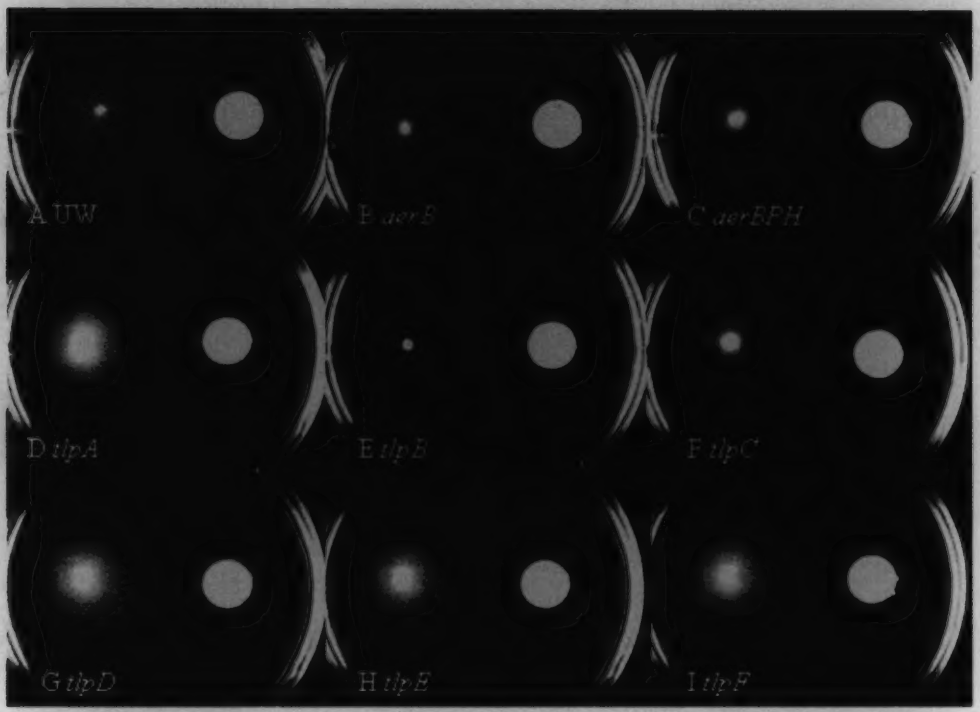

Figure 3.22 Chemotactic patterns formed by various strains of *A. vinelandii* around discs soaked in 10 mM ammonium chloride

CHAPTER 4

DISCUSSION

4.1 Discussion of bioinformatics, and transducer mutant work

The chemotaxis, energy taxis and motility related genes in several prokaryotic genomes have already been described; this work describes for the first time these genes in the *A. vinelandii* UW genome. The identification and analysis of genes involved in taxis is an excellent initial step to elucidating the motile behaviour of *A. vinelandii*. The identification of taxis transducer proteins in particular, is a very powerful means to glean insight into the chemicals, and physiochemical parameters that may govern motility.

The signal transduction system, the *che* genes, in the *A. vinelandii* genome is complete and it contains all the major components that are found in *E. coli* (Figure 3.1; Stock, 1996). This result was not unexpected as nearly all motile bacteria and archaea share a similar chemosensory signalling system (Alexandre *et al.*, 2004). There is only one copy of the taxis signal transduction proteins in the *A. vinelandii* genome, which is the same as *E. coli* and *B. subtilis* (Rao *et al.*, 2004) but unlike other bacteria such as *R. sphaeroides* and *S. meliloti* (Armitage & Schmitt, 1997), which possess multiple copies of one or more components of the Che signal transduction system.

A. vinelandii has a complement of nine taxis transducers through which it can potentially sense its environment. The two energy taxis systems, AerPH and AerB, in *A. vinelandii* were identified as energy taxis transducers because of the PAS domains present in AerP and in the N-terminal region of AerB. The PAS domains in these proteins may bind a redox-responsive cofactor, such as FAD, and interact with the electron transport chain to monitor flow of electrons through the electron transport chain, as originally proposed by Taylor *et al.* (1999). The energy taxis systems AerB and

AerPH are orthologues of Aer in *E. coli* (Bibikov *et al.*, 1997), Aer in *P. putida* (Nichols & Harwood, 2000), and CetAB in *C. jejuni* (Hendrixson *et al.*, 2001), and may function in a similar fashion.

The *in silico* analysis of the other six transducers; TlpA, TlpB, TlpC, TlpD, TlpE and TlpF, revealed no motifs or any other homology within the periplasmic region that would indicate their sensing capabilities. However the presence of these six alternative transducers suggests that *A. vinelandii* is capable of sensing an array of specific substrates in addition to whatever physiochemical parameters may be sensed by AerB and AerPH.

A comparative analysis of the *Azotobacter* energy taxis transducers to PAS-bearing energy taxis transducers from other species revealed a high identity among the *Azotobacter* transducers. See Table 3.1 for a complete listing of all transducers used in this study. Phylogenetic analysis reveals that AerPH and AerB are more closely related to one another than they are to transducers from other organisms. This close relationship suggests that a gene duplication event occurred at some point in the history of the *A. vinelandii* genome. Furthermore the discovery of a fully intact transducer gene, *aerS*, between *mutS* and *rpoS* in *A. salinestris* highlights the variability of this region among Azotobacter species, a phenomenon identified in some enteric genomes (Herbelin *et al.*, 2000; Kotewicz *et al.*, 2003). The description of *aerPH* and *aerS* between the *rpoS mutS* region is the first description of transducer sequences found in this polymorphic locus. It has been postulated that both *mutS* and *rpoS* are subject to frequent mutation in bacterial evolution and that the acquisition of sequences between these two genes is related to their high rates of loss and restoration over time (Ferenci, 2003). These energy taxis genes in the *mutS-rpoS* region are not present in all *Azotobacter* species; for example in *A.*

chroococum and in *A. macrocytogenes* only *fdxA* is present (D Meakins and J. Robitaille). It is possible that the acquisition of an energy taxis transducer in the *rpoS-mutS* region provided an advantage to *A. vinelandii* and *A. salinestris*.

In general most of the other bacterial and archael PAS-bearing energy taxis transducers examined in this study share the same domain organization and composition as AerB of *A. vinelandii* and Aer of *E. coli* (Bibikov *et al.*, 1997). There are some notable exceptions, including a large number of transducers that contain no apparent transmembrane domain (Table 3.1). There is as of yet no literature describing how these cytoplasmic energy taxis transducers function. There is a trend for the cytoplasmic transducers to contain multiple copies of the PAS and PAC domain (Table 3.1). It has been suggested that the multiple copies of the PAS and PAC domains may amplify the sensory signal (Zhulin & Taylor, 1998). Perhaps the cytoplasmic location of these transducers requires a more complex sensory region that offers a graded output capable of detecting subtle changes in the intercellular energy level.

There were three other bipartite energy taxis systems, in addition to AerPH, that were identified in the sequences databases. These energy taxis systems are characterized as adjacent ORFs encoding PAS and HCD domains, respectively. Only the CetAB system from *C. jejuni* has been demonstrated to be functional (Hendrixson *et al.*, 2001) it is therefore interesting to note that the AerPH system is most distantly related from *C. jejuni* compared to *P. resinovorans* and *C. violaceum* (Figure 3.5).

The search for PAS-bearing energy taxis transducers in the SMART, Swiss-Prot , TrEMBL and JGI sequence databases generated a variety of transducers from gram-negative bacteria and several archaea, but no gram-positive bacteria. This may be due to

a bias in the sequences available on the databases or perhaps motile gram-positive organisms, in general, employ Heme-based transducers to sense oxygen gradients, as is the case for *B. subtilis* (Hou *et al.*, 2000).

The investigation of the PAS-bearing energy taxis transducers has theoretical implications in understanding the evolutionary history of this small branch of a remarkable family of proteins. It should be noted that the PAS domain, common to all of these transducers, may or may not contain a FAD cofactor like Aer of *E. coli*. PAS domains are known to bind other cofactors and the vast majority of these transducers have not been investigated to determine the prosthetic group bound by the PAS domain.

These transducers were identified on *A. tumefaciens* Ti plasmids, megaplasmids from *R. meliloti* and *R. solanacearum*, on the transposable element Tn*1721* in *E. coli*, and in the polymorphic *mutS-rpoS* region of the chromosome in *A. vinelandii* and *A. salinestris*. It is possible that these transducers have undergone one or more lateral transfer events. However these transducers arose within the different gram-negative and archael genomes it appears that they are a common component which motile cells use to sense their environment.

The soft agar assay demonstrated that for *A. vinelandii* UW anything that could be used as an energy source would generate swimming halos. The major exceptions to this observation were citrate and oxaloacetate (Table 3.2). Although these two compounds could apparently be used as a carbon sources (Thompson & Skerman, 1979) they were not good substrates for motility in the soft agar assays.

The examination of the *aerB* and *ΔaerPH* mutants in soft agar media identified a role for AerB but not for AerPH. In the soft agar medium the cells metabolize the carbon

source at the point of inoculation creating a natural gradient of the carbon source to which the cells respond, at the same time the cells consume dissolved oxygen in the medium and create a gradient of oxygen. It is not always possible to distinguish which of the two dual gradients of carbon compound (electron donors) and oxygen (terminal electron acceptor) the cells are responding to, however all the strains are subject to the same conditions and any difference in halo appearance can be attributed to the transducer mutation. The *aerB* mutant had a reduced halo diameter on all media tested; furthermore the appearance of the swimming halos had a more defined edge than the UW halos. The triple *aerB,PH* mutant performed identically to the *aerB*- mutant, giving no indication to a function for AerPH. It is likely that the stop codon between the *aerP* and *aerH* genes resulted in a loss of function of these genes, and that they do not function co-ordinately, unlike CetAB of *C. jejuni*. Another explanation is that AerPH still may function together but they are only expressed under certain conditions and these were not achieved under the experimental conditions. It is possible that at one point the *aerPH* genes were not interrupted by a stop codon, like *aerS* of *A. salinestris*, and may have been a functional energy taxis transducer at that time. Current research is underway to determine if *aerS* of *A. salinestris* and *aerB* from UW can be placed onto a broad host range vector and transformed into the *aerB* mutant to complement the non-energy taxis phenotype (H. Whelan and J. Robitaille, unpublished). One or more of the other classical taxis transducer mutants may be able to sense oxygen or energy, as the *aerB* and *aerB,PH* mutants still produce halos on soft agar media. This is not unexpected as Tsr of *E. coli* has been implicated in energy sensing (Rebbapragada *et al.*, 1997).

The aerotaxis assays indicate that AerB is able to guide the cells in a spatial gradient of oxygen. When *aerB* is disrupted the cells respond as though they are blind to oxygen, over time forming a band of non-motile cells at a specific distance away from the air-liquid interface. Without AerB to signal to the chemosensory machinery to bias the cells direction towards oxygen they will, through random chance, swim away from their optimal oxygen concentration. This is a deleterious behaviour, because as an obligate aerobe *A. vinelandii* must have oxygen to generate a proton motive force. If a cell moves to a microenvironment without sufficient oxygen available it is no longer able to generate enough energy to rotate its flagella and consequently becomes non-motile. This effect is noticeable when observing cells under a microscope- a glass cover slip over the cell suspension will render the cells non-motile, once removed the cells will swim again.

Aerokenisis, speed acceleration towards oxygen concentrations, was observed by *A. vinelandii* in the aerotaxis assays. This behaviour has been described in the soil bacterium *S. meliloti* (Zhulin *et al.*, 1995). Speed changes are a result of flagellar rotation which is driven by Δp. *S. meliloti* and *R. sphaeroides* can alter the rotation of their flagella even when Δp is considered to be saturating, *E. coli* has not been demonstrated to share this ability (Armitage & Schmitt, 1997). Interestingly the only other study on *A. vinelandii* motility demonstrated that the cell's speed increased in higher concentrations of chemoattractants (Haneline *et al.*, 1991). Taken together these results indicate that alterations in speed may be a general characteristic of *A. vinelandii* motility.

Studies on the six classical transducer mutants identified TlpB as an important factor in the tactic response of *A. vinelandii*. The *tlpB* mutant produced much smaller halos in all media tested (Figure 3.9), suggesting it may be another energy taxis

transducer, like Tsr in *E. coli*. In many cases the *tlpB* mutant produced much smaller halos than the *aerB* mutant. Future work may involve constructing a double *aerB tlpB* mutant and testing the effects of these mutations on motility. TlpC is also implicated in sensing organic acids as it produces notably smaller halos than UW on ammonium acetate, sodium acetate, and succinate compared to glucose and glycerol (Figure 3.9). Organic acids such as TCA cycle intermediates are common carbon sources in soil environments and are preferred carbon sources for *A. vinelandii* (George *et al.*, 1985). It is not surprising at least one of the transducers appears to guide *A. vinelandii* towards organic acids. TlpD may play a role in sensing glycerol, because the *tlpD* mutant produced smaller halos than UW on the glycerol medium.

A comparison of the swimming halos on sodium acetate and ammonium acetate media was done to compare the motility of the different strains under nitrogen fixing and non-nitrogen-fixing conditions. The only strain that had an obvious difference in performance was *tlpA*, which produced larger halos in the non-nitrogen fixing conditions (Figure 3.9). The *tlpA* mutant performed markedly better than UW on glycerol, sorbitol and sodium acetate. It is not clear from these data what TlpA senses, but when it is absent the mutants are able to swim further, suggesting it may repress running cues or stimulate swimming cues under certain conditions. Similarly the *tlpE* and *tlpF* mutants produced larger halos on ammonium acetate, sodium acetate and succinate.

The first and only description of *A. vinelandii* chemotaxis in the literature was by Haneline *et al.* (1991). In that work the researchers demonstrate that *A. vinelandii* can respond chemotactically to gradients of many carbohydrate compounds. Chemotaxis was inducible to certain compounds by pre-growing the cells on those chemicals. This work

expands upon that original research by identify and mutating the transducers responsible for sensing chemicals and physiochemical parameters. The soft agar work does not distinguish between aerotactic responses and chemotactic responses. In order to adequately investigate the response of *A. vinelandii* to chemicals direct microscopic observation is important. The best approach would be to use a tethered cell assay, where the flagella are tethered to a glass slide and observed microscopically. Different chemicals can be added and the rotation of the tethered cells can be observed. This experiment may also help identify chemokenesis, if it is indeed an important factor in the motile behaviour of *A. vinelandii*. In addition these experiments would identify whether the flagella in *A. vinelandii* are capable of changing rotation, like *E. coli*, or are only capable of rotating in one direction, like *S. meliloti* (Armitage & Schmitt, 1997).

Transducers proteins are known to sense a variety of stimuli, making them highly variable sensors. Tsr in *E. coli* has been implicated in sensing serine, external pH, weak acid repellents, temperature, hydrophobic amino acids and indole (Stock, 1997; Stock, 1996). Likewise the transducers in *A. vinelandii* may sense a variety of stimuli and two or more transducers may sense redundant cues, making characterization of the transducers a challenge. There is no data indicating the expression of these transducers in *A. vinelandii* or indicating their relative abundance. There is growing evidence that transducers in bacteria are clustered at the poles of the cells and function cooperatively to sense, amplify and transmit environmental cues to the cell (Bray *et al.*, 1998; Sourjik & Berg, 2004). It is unclear how the nine transducers in *A. vinelandii* cooperate to integrate a signal to affect flagellar rotation. Are there transducers in *A. vinelandii* whose signal dominates other transducers? Certainly AerB is a candidate as a dominant transducer due

to the essential role of oxygen in *A. vinelandii* metabolism and the consequences for a cell that finds itself in an oxygen-depleted microenvironment.

The study of taxis transducers in *A. vinelandii* had identified two transducers, AerB and TlpB, which are very important proteins in the sensory-motor pathway of this organism. TlpB is a new transducer to add to the list of classical transducers, like Tsr, that may be involved in energy sensing. The other predicted energy taxis system AerPH had no known function and is likely a non-functional artefact of an earlier transducer. A putative role in sensing organic acids was identified for TlpC and glycerol for TlpD. This work is a starting point in understanding how these remarkable proteins function in *A. vinelandii* and opens new directions for research.

4.2 Discussion of chemotactic aggregation and pattern formation by motile *A. vinelandii*

The discovery of chemotactic aggregation of *A. vinelandii* was a serendipitous result that arose from experiments examining the motility of this organism in the presence of amino acids. It is now known that the soft agar medium composition that initially yielded autoaggregation was a product of the sterilization process. Asparagine, at high temperatures, is likely broken down into ammonia and acrylic acid (Figure 3.13). It is the ammonia that is believed to be responsible for initiating the autoaggregation behaviour. This conclusion is supported by the result that aggregates form around ammonium-soaked discs. Acrylamide, another breakdown product of asparagine also results in autoaggregation, although the arrangement of the aggregates is less symmetrical

than the autoclaved asparagine or ammonia patterns. It is believed that the chaotic appearance of the acrylamide patterns is due to the deamination of acrylamide that generates intermittent gradients of ammonium; therefore the aggregates are also arranged at irregular intervals. Experiments in which ammonia is added directly to the soft agar media has so far not yielded aggregates. Therefore a more complex interaction between the cells and the medium (acrylic acid) maybe responsible for yielding aggregates.

A common factor in all the experiments is the dependence of the autoaggregation behaviour on the initial concentration of autoclaved asparagine, acrylamide or ammonia. An optimal concentration of 2.5 mM asparagine reproducibly yielded autoaggregates. The ammonium-soaked disc assays clearly demonstrated the dependence of autoaggregation on concentration of ammonia. The aggregates only formed at a very specific distance from the discs; moreover the appearance of the aggregates was different depending on their proximity to the disc. The concentration of ammonia in the disc can be altered to manipulate the pattern of autoaggregates formed around the discs (Figure 3.21).

In addition to nitrogen source there were several other variables that were identified to be important in pattern formation. The cells were always supplied with glucose as a metabolizable carbon source. A range of glucose concentration was examined and found to have little effect on the appearance of the patterns, however low glucose concentrations (10-30 mM) yielded patterns that were unstable and they tended to cease aggregation, for this reason glucose concentrations were kept high (90- 100 mM) so that the cells respond to other factors, not a gradient of glucose. These observations

suggest while glucose is essential as an energy source for the cells does not drive the aggregation behaviour.

The agar concentration is another variable important in pattern formation. The goal of the soft agar plates is to create an environment that is liquid enough to allow the cells to swim through, but solid enough to prevent excessive diffusion of the chemicals within the media. It was determined that an agar concentration of 0.24% to 0.28% was optimal for generation of patterns in these experiments. Any deviation form these agar concentrations had a profound effect on autoaggregation and pattern formation (Figure 3.16). From these results it can be speculated that the concentration of specific chemicals within the media is an essential factor in determining whether cells will aggregate.

Aggregation was observed only when very high cell densities were used to inoculate the soft agar plates. It may be that high cell densities are able to generate gradients of certain chemicals more rapidly than low cell densities and that these steep chemical gradients are necessary for aggregation. Similarly there may be cellular activities that are only carried out at high cell densities that are essential for triggering aggregation. In *E. coli* and *S. typhimurium* saturated overnight cultures were used to generate the complex patterns (Blat & Eisenbach, 1995; Budrene & Berg, 1991, respectively). However in *Pseudomonas* strain KC cultures with an OD_{600} of 0.6 were used to inoculate semi-solid agar to produce the patterns. Therefore the requirement of a very high cell density does not appear to be a universal requirement for motile cells to autoaggregate.

The autoaggregates themselves are macroscopic and develop over time. Microscopic examination can detect no clear morphological differences between the cells

within the aggregates and free-swimming cells. The aggregates develop radially over time in a symmetric fashion. In their investigation of the dynamic formation of the patterns produced by chemotactic *E. coli* Budrene & Berg (1995) observed the aggregates developing behind an expanding ring. This phenomenon was not clearly observed in the *A. vinelandii* patterns, as there was no clear expanding ring behind which the aggregates formed. New rows of aggregates developed sequentially over time between aggregates in the previous row. The aggregates once formed were very stable; they did not disperse, as has been described for aggregates formed by *E. coli* (Budrene & Berg, 1995) and *S. typhimurium* (Blat & Eisenbach, 1995).

There were unique patterns produced by *A. vinelandii* that have not been described in other organisms. In certain experiments the cells did not form discrete spots, but continued to swim in long stripes several centimeters in length (Figure 3.19B). These patterns were difficult to reproduce and the specific conditions required to generate the stripes remains unknown. However it is likely that subtle changes in the initial composition of the medium will determine the length of the aggregates. The aggregates formed in the ammonium soaked disc assays are longer in length the further their distance from the disc. These data suggest that the appearance of the aggregates is highly dependent upon the concentration of ammonia in the medium. The method of preparing the autoclaved asparagine is prone to inconsistency and this is likely the main cause of the inability to reproduce the long striped patterns successfully.

The asparagine-generated patterns do not develop between two adjacent inoculation points; they appear to repel one another and develop in relatively straight lines. This effect is diminished as acrylamide is added to the medium (Figure 3.18). The

reasons behind this repelling effect are unknown, but it does indicate a complex sensory mechanism behind the patterns. It also suggests that the mechanism behind the acrylamide generated patterns is different than the asparagine-generated patterns.

From these data a model for aggregation and pattern formation can be proposed. The autoaggregation in *A. vinelandii* is likely a result of a motile response of the cells towards an attractant which they themselves excrete. This attractant, which is currently unknown, is expected to be produced when the cells encounter the substrate ammonium. This is the basic model that has been proposed for pattern formation by *E. coli* by (Budrene & Berg, 1995) and is likely responsible for the complex patterns formed by *S. typhimurium* (Blat & Eisenbach, 1995) and *Pseudomonas* strain KC (Emerson, 1999). Mathematical modeling of these patterns in *E. coli* can accurately predict the patterns if they take into account the capacity of the cells to produce a potent chemoattractant (Brenner *et al.*, 1998; Tyson *et al.*, 1999). Initial mathematical modeling of the *A. vinelandii* patterns is also reliant upon the assumption that cells excrete a potent chemoattractant (T. Hillen personal communication). What induces the cells to begin excreting this ammonia-dependent attractant is unknown, however it may require a high cell density and it may be dependent on external ammonium concentration.

This is the first example of a nitrogen-fixing organism autoaggregating in response to a nitrogen source. The question becomes what role, if any, does diazotrophy, play in the aggregation behaviour? In the ammonium-soaked disc assays the initial inocula are fixing nitrogen and will do so until they encounter a fixed nitrogen source, in this case ammonium. It is likely that the cells, upon forming an autoaggregate, switch from a diazotrophic to ammonium-assimilating response. Is this metabolic shift sensed

by any of the transducers and how does it affect the growth and pattern of the autoaggregates?

There are many factors that determine whether *A. vinelandii* will express nitrogenase, these include: energy, carbon, oxygen and fixed nitrogen. These factors are sensed by the atypical sensor kinase NifL (Dixon, 1998). NifL is responsive to the fixed nitrogen status of the cell and the ADP levels in the cell. If either of these two parameters are high then NifL will repress its cognate response regulator NifA thereby repressing the transcription of the nitrogenase genes. NifL encodes a PAS domain that contains a redox responsive FAD cofactor, very similar to the proposed sensing domain of AerB. If NifL becomes oxidized it blocks the activity of NifA, (Dixon, 1998). The redox potential of the FAD prosthetic group in NifL is –196.7 mV (Dixon, 1998). If AerB is in fact a flavoprotein it would be interesting to compare the redox potential of FAD in AerB with NifL.

The transducer knockout mutants were examined for their ability to produce the autoaggregates around ammonium-soaked discs. Four transducers are required to produce autoaggregates; AerB, TlpA, TlpB, and TlpC (Figure 3.22). The other transducers do not appear to be necessary for pattern formation, as the knock out mutants produced the patterns identical to the wild type UW cells. The Δ*aerPH*, *aerH*, and *aerP* mutants were also able to produce patterns like UW. The requirement of functional transducers TlpA, TlpB, TlpC and AerB can be supported by the excreted chemoattractant model. At least one of the transducers must be required to sense the excreted attractant, TlpA and TlpC are good candidates for this function as they do not appear to be general energy sensing transducers. In addition one of these transducers

ought to guide the cells in gradients of ammonium, which is analogous to the model for pattern formation in *E. coli* in response to aspartate. Since the results suggest that AerB and TlpB are transducers for energy taxis, the observation that they are both necessary for pattern formation suggest that energy sensing is an important feature in production of the aggregates. These results compare with previous results for *E. coli* which identifies the taxis transducer Tar as essential for autoaggregation (Budrene & Berg, 1991). The *S. typhimurium* Tar protein is necessary for aggregation, but only under certain conditions (Blat & Eisenbach, 1995). Both *E. coli* and *S. typhimurium* have Aer homologues, it would be interesting to examine whether those transducers are important for aggregation.

There is one important paradox to the phenomenon of autoaggregation observed in *A. vinelandii*. As an obligate aerobe *A. vinelandii* is absolutely dependent on oxygen for energy generation and, as observed earlier, rapidly becomes non-motile in anoxic conditions. Within the dense cellular aggregates the individual cells are all respiring and likely use up the oxygen rapidly. In the aggregates produced by *Pseudomonas* strain KC it was demonstrated that there was little or no oxygen in the aggregates (Emerson, 1999). The question then arises as to the benefit for *A. vinelandii* to aggregate to such densities if it results in the creation of an anoxic environment. It may be that the cells within the aggregates are in an oxygen-deprived state of non-motility, which would explain the stability of the aggregates. Perhaps only cells on the edge of the aggregates have access to the oxygen that allows them to escape the cell cluster, only to repeat the cycle over again. Despite this detrimental effect of cell clustering one can speculate as to the benefits that a group of autonomous cells may have in clustering together. Although quorum sensing has been studied in many bacterial species (for a review see Miller &

Bassler, 2001) is has never been identified in *A. vinelandii*. Genomic searches yield no hint of any genes that maybe involved in this process. If some novel form of quorum sensing is used by *A. vinelandii* to communicate and coordinate activities at high cell densities it is certainly possible that cells in aggregates are candidates for participating in this process.

There are a number of potential benefits to the cells for aggregating together including collective defence, biofilm formation, genetic exchange, or even protection of nitrogenase from oxygen. The protection of nitrogenase from oxygen is a paradoxical speculation as the cells appear to aggregate in response to nitrogen sources and therefore may not be fixing nitrogen. The patterns, though remarkably complex and demonstrative of multicellular behaviour may be nothing more that laboratory induced artefacts. The natural soil or freshwater habitat that *A. vinelandii* are found is very difficult to simulate using soft agar media and therefore there may be many other conditions in the environment that will enable cells to autoaggregate. The chemosensory machinery governing taxis is very complex and this research only touches the surface of this behaviour in *A. vinelandii*. This initial work has shown that *A. vinelandii* can use its taxis machinery to govern the cells to aggregate in response to one another, and may be part of a sophisticated intercellular communication strategy.

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